Pre-Summer Preparation work

- Was introduced to the Bowman-Birk Inhibitor(BBI) by Dr. Aaron Goodarzi, who is a radiooncology researcher at the Charbonneau Cancer Institute at the University of Calgary
    - BBI: a protease inhibitor derived from soybeans, which was found to have radioprotective properties by a lab in Germany headed by Dr. Klaus Dittman (Dittmann K, Loffler H, Bamberg M, Rodemann HP. Bowman-Birk proteinase inhibitor (BBI) modulates radiosensitivity and radiation-induced differentiation of human fibroblasts in culture. Radiother. Oncol. 1995;34:137–143.)
- Decided on doing the BBI as our radioprotective peptide of choice; found the sequence of the truncated or modified version of the BBI which has been shown to retain its functions as a radioprotector (Dittmann KH, Gueven N, Mayer C, Rodemann HP. Characterization of the amino acids essential for the photo- and radioprotective effects of a Bowman-Birk protease inhibitor-derived nonapeptide. Protein Engineering. 2001;14:157–160.)
    - The whole BBI protein (70 a.a.) can cause problems in blood clotting; 9 a.a. Sequence avoids that issue, because the section of the peptide that acts as a protease inhibitor is cut off
- Previous literature has also suggested a potential molecular pathway by which BBI works. (Gueven N, Dittmann K, Mayer C, Rodemann HP. Bowman-Birk protease inhibitor reduces the radiation-induced activation of the EGF receptor and induces tyrosine phosphatase activity. Intl. J. Radiat. Biol. 1998;73:157–162.) BBI mostly works in Non-Homologous End Joining pathways, which is expected as that is the most prevalent mode of DNA double stranded repair in cells
- Looked into other potential peptides that we can use in case the BBI does not work out: KTI, Glutathione, antioxidants
    - KTI: another radioprotective peptide that has been found to be effective in protecting against certain types of radiation that the BBI does not (Van den Hout, R.; Pouw, M.; Gruppen, H. Inactivation kinetics study of the Kunitz soybean trypsin inhibitor and the Bowman−Birk inhibitor. J. Agric. Food Chem. 1998, 46 (1), 281−285.)
    - Glutathione: a reactive oxygen species “sink” that quenches reactive oxygen species by binding to its lone electron. Mostly works near the mitochondria of the (Chatterjee A. Reduced Glutathione: A Radioprotector or a Modulator of DNA-Repair Activity? *Nutrients*. 2013;5(2):525-542. doi:10.3390/nu5020525.)
    - Antioxidants: works by a variety of pathways (Weisse J. F. and Landauer M. R., Radioprotection by Antioxidants. *Annals of the New York Academy of Sciences.* 2000; 899: 44–60. doi:10.1111/j.1749-6632.2000.tb06175.x)
- Also looked at current other methods of radioprotection (doubles as market research for the application of our device) (Kamran, M. Z., Ranjan, A., Kaur, N., Sur, S. and Tandon, V. (2016), Radioprotective Agents: Strategies and Translational Advances. Med. Res. Rev., 36: 461–493. doi:10.1002/med.21386)
    - Currently some radioprotectors have been proposed for cancer radiotherapy (ex. synthetic thiol-containing compounts, amifostine) but uptake by the public and medical community has not been great as side-effects were undesirable and drug benefits were not well defined
    - NASA Biocapsule - we did not know about this the time when we came up with our project, but after some searching around we stumbled apon this on the internet. Comparable technology to our device, unsure about the progress/research that has been done on this as NASA is very secretive (http://sservi.nasa.gov/articles/nasa-breakthrough-could-save-millions-lives/; http://www.medgadget.com/2012/02/nasa-biocapsule-implant-diagnoses-and-treats-diseases-without-human-intervention.html)
- Were unsure about the specific potential application of our peptide: it was a debate between cancer radiation prevention (as radiotherapy is known to cause secondary cancers, and certain existing literature stated that BBI has radioprotective effects only on p53+ cells, which about 60% of all cancers are p53-) (Dittmann KH, Gueven N, Mayer C, Ohneseit P, Zell R, Begg AC, Rodemann HP. The presence of wild-type TP53 is necessary for the radioprotective effect of the Bowman-Birk

proteinase inhibitor in normal fibroblasts. Radiation Research. 1998;150:648–655.), or space application
    - We realize these applications would call for very different designs for our device as well as different methods of testing, but those differences exist mostly in the more refined details of our project, which would not affect our preliminary work
    - Decided that we needed a deadline by which to decide the application of our project: End of June (tentative)
- Planned out some experiments we wanted to do to test viability of the various peptides: Clonogenic survival assay with both cancer cells as well as primary cells
    - We had a few variations on the clonogenic survival: thought about doing high throughput assays with 96 well plates, dual plating with cancer and primary cells to see interactions, comparison of survivability of cancers vs. primary cells with and without BBI treatment
- Expected results: BBI is expected to protect our primary cells against radiation while not protecting our cancer cells as much as the primary cells. Meaning that the surviving fractions of our cell lines treated with BBI will be higher than that of cell lines not treated with BBI, and the surviving fractions of primary cell lines treated with BBI will be higher than that of cancer cell lines treated with BBI

May 3, 2016

Dr. Goodarzi Interview

- We met with Dr. Goodarzi to talk about potential assays and our prospects for the summer
- Dr. Goodarzi confirmed the viability of our clonogenic survival assay in testing the effectiveness of BBI and mBBI, but he also suggested a few more assays we can use, namingly the H2AX assay (Mariotti LG, Pirovano G, Savage KI, Ghita M, Ottolenghi A, Prise KM, et al. (2013) Use of the γ-H2AX Assay to Investigate DNA Repair Dynamics Following Multiple Radiation Exposures. PLoS ONE 8(11): e79541. doi:10.1371/journal.pone.0079541) and the Flow cytometry assay
    - H2AX: Can use fluorescent tags to tag H2AX foci (histone proteins that localize around double stranded breaks) to detect the number of double stranded breaks post irradiation with or without BBI. Using fluorescent microscopy to view double stranded breaks post irradiation, we can blind count the number of detected foci at various timepoints within 24 hours of irradiation to see if the cells treated with BBI are able to repair their double stranded breaks (marked by the decrease of fluorescent foci) at a faster rate comared to cells that are not
- Dr. Goodarzi had also suggested that we use non-cancer cell lines as well as cancerous cell lines to test the difference in radiation protection in cancer cells versus non-cancerous cell lines
- Listed a number of cell lines that will be able to provided for us, including a variety of cancerous cell lines as well as normal cell lines

May 4, 2016

Laboratory introduction and techniques week

- Made and Autoclaved agar (1L)
    - 1% tryptone (10g)
    - 0.5% Yeast extract (5g)
    - 1% NaCl (10g)
    - 1.5% Agarose (15g)
- Poured plates of LB agar with 3 different types of antibiotics:
    - Amp (stock 100 mg/mL; final 100 ug/mL)
    - Kan (stock 50 mg/mL; final 50 ug/mL)
    - Chlor (stock 50 mg/mL; final 30 ug/mL)

May 5, 2016

Laboratory introduction and techniques week

- Results: Cultured competent E.Coli as well as Chlor-resistant E.Coli on each type of Agar plate(s)
    - Growth of a lawn of red-coloured bacteria (Chlor-resistant culture on Chlor plate & Chlor-resistant culture on Amp plate)
    - Growth of a lawn of white/yellow- coloured bacteria (competent E.Coli culture on Amp plate) --> contamination (?)
    - No growth observed on other plates
- Results: Liquid Culture of normal as well as Chlor-resistant E.Coli (no antibiotic added to liquid culture)
    - Growth of white/yellow bacterial cultures on the bottom of all tubes
- Made 50mM $CaCl_2$ (5mL of 1M $CaCl_2$, 95mL ddH_2O) & 50mM $CaCl_2$ with 15% Glycerol (5mL 1M $CaCl_2$, 15mL Glycerol, 80mL ddH_2O) for making competent E.Coli --> placed in 4°C fridge
- Inoculated 2 tubes of 5 mL LB with old (#10) competent *E. coli* cells
- Inoculated 2 tubes of 5 mL LB with new (C3037) competent *E. coli* cells (Used steril 5 mL LB as negative control) --> incubated at 37°C O/N, shaking (200 rpm)

May 6, 2016

Laboratory introduction and techniques week

- The overnight cultures of competent *E. Coli* (#10 and C3037) were subcultured in 49ml of LB. There were four subcultures total: 2xC3037 and 2x#10. Cultures were incubated at 37.5°C and shaken at 200rpm for 4 hours. The cultures were grown to an optical density of 0.647 (within the range of 0.4-0.6)
- Chemical comptenece
    - Cultures were spun down for 7 minutes at 3000rpm and 4°C
    - 12.5ml of cold 50mM $CaCl_2$ was added and the pellet resuspended
    - Cells were then incubated on ice for 10 minutes
    - Cells were spun down again at 1500rpm for 2 minutes at 4°C
    - Cells were resuspended in 2ml of 50mM $CaCl_2$ with 15% glycerol
    - Transfer to microcentrifuge tube in 100µl aliquots
    - Storage at -80°C
- The rest was left up to chassis team to document and discuss results

May 9, 2016: EXPERIMENTAL GOALS FOR THE SUMMER - DISCUSSION

BBI Efficiency
- test in human cell lines
- requires positive/negative control

Cytotoxic treatment
- induce DNA damage from something other than radiation/determination of mechanism
- H2AX assay

Circuit Function
- testing construct components
- promoters and RBS
- test with reporter genes
- BBI expression
- use mass spec/MALDI to measure peptide amount
- use rtQ-PCR as a backup

Excretion (overlap with Chassis)
- similar assay to BBI excretion

May 10, 2016

- Researched the demand for a radioprotective strategy in space exploration, found that NASA and the CSA are trying to further research efforts in this area, making it an important issue to address (http://www.space.com/21353-space-radiation-mars-mission-threat.html)
- Similar products are currently under development by NASA called the biocapsule (http://www.americaspace.com/?p=13700)
    - The developments of the biocapsule technology is unknown in the public, so we do not know how similar the biocapsule is from our proposed project

May 11, 2016

Peptide Sequences

- Compiled the mBBI amino acid sequences found in literature searches, and also put in some of our own additions to prime the peptide for secretion and delivery, as well as to accommodate our own assays
- Reverse translated them into genetic sequences for E.Coli as well as B. Subtilis

Common Identifier	FASTA	E. Coli	B. Subtilis	D. Radiodurans
(S) 9mer	CALSYPAQC	TGC-GCG-CTG-AGC-TAT-CCG-GCG-CAG-TGC	TGC-GCA-CTG-TCA-TAT-CCG-GCA-CAA-TGC	UGC-GCC-CUG-AGC-UAC-CCC-GCC-CAG-UGC
(V) 9mer	CALVYPAQC	TGC-GCG-CTG-GTG-TAT-CCG-GCG-CAG-TGC	TGC-GCA-CTG-GTT-TAT-CCG-GCA-CAA-TGC	UGC-GCC-CUG-GUG-UAC-CCC-GCC-CAG-UGC
(S) 7mer	ALSYPAQ	GCG-CTG-AGC-TAT-CCG-GCG-CAG-TGC	TGC-GCA-CTG-TCA-TAT-CCG-GCA-CAA-TGC	GCC-CUG-AGC-UAC-CCC-GCC-CAG
BBI protein	SACKSCICALSYPAQCFCVDIT	UCC-GCG-UGC-AAA-UCC-UGC-AUU-UGC-GCG-CUG-UCC-UAU-CCG-GCG-CAG-UGC-UUU-UGC-GUG-GAU	TCA-GCA-TGC-AAA-TCA-TGC-ATT-TGC-GCA-CTG-TCA-TAT-CCG-GCA-CAA-TGC-TTT-TGC-GTT-GAT-ATT-ACA	
Peptide #1 BBI	KSCICALSYPAQCF	AAA-AGC-TGC-ATT-TGC-GCG-CTG-AGC-TAT-CCG-GCG-CAG-TGC-TTT	AAA-TCA-TGC-ATT-TGC-GCA-CTG-TCA-TAT-CCG-GCA-CAA-TGC-TTT	AAG-AGC-UGC-AUC-UGC-GCC-CUG-AGC-UAC-CCC-GCC-CAG-UGC-UUC
Peptide #2 - NLS	GPKKKRKVKSCICALSYPAQCF	GGC-CCG-AAA-AAA-AAA-CGC-AAA-GTG-AAA-AGC-TGC-ATT-TGC-GCG-CTG-AGC-TAT-CCG-GCG-CAG-TGC-TTT	GGC-CCG-AAA-AAA-AAA-AGA-AAA-GTT-AAA-TCA-TGC-ATT-TGC-GCA-CTG-TCA-TAT-CC	GGC-CCC-AAG-AAG-AAG-CGC-AAG-GUG-AAG-AGC-UGC-AUC-UGC-GCC-CUG-AGC-UAC-CCC-GCC-CAG-UGC-UUC

			G-GCA-CAA-TGC-TTT	
Peptide #3 BBI HA	KSCICALSYPAQCFYPYDVPDYA	AAA-AGC-TGC-ATT-TGC-GCG-CTG-AGC-TAT-CCG-GCG-CAG-TGC-TTT-TAT-CCG-TAT-GAT-GTG-CCG-GAT-TAT-GCG	AAA-TCA-TGC-ATT-TGC-GCA-CTG-TCA-TAT-CCG-GCA-CAA-TGC-TTT-TAT-CCG-TAT-GAT-GTT-CCG-GAT-TAT-GCA	AAG-AGC-UGC-AUC-UGC-GCC-CUG-AGC-UAC-CCC-GCC-CAG-UGC-UUC-UAC-CCC-UAC-GAC-GUG-CCC-GAC-UAC-GCC
Peptide #4 BBI NLS-HA	GPKKKRKVKSCICALSYPAQCFYPYDVPDYA	GGC-CCG-AAA-AAA-AAA-CGC-AAA-GTG-AAA-AGC-TGC-ATT-TGC-GCG-CTG-AGC-TAT-CCG-GCG-CAG-TGC-TTT-TAT-CCG-TAT-GAT-GTG-CCG-GAT-TAT-GCG	GGC-CCG-AAA-AAA-AAA-AGA-AAA-GTT-AAA-TCA-TGC-ATT-TGC-GCA-CTG-TCA-TAT-CCG-GCA-CAA-TGC-TTT-TAT-CCG-TAT-GAT-GTT-CCG-GAT-TAT-GCA	GGC-CCC-AAG-AAG-AAG-CGC-AAG-GUG-AAG-AGC-UGC-AUC-UGC-GCC-CUG-AGC-UAC-CCC-GCC-CAG-UGC-UUC-UAC-CCC-UAC-GAC-GUG-CCC-GAC-UAC-GCC

May 12, 2016

- Asked for peptide quotes from a multitude of peptide synthesis companies to find the cheapest and best synthesis deal

May 14-15, 2016: Lethbridge Workshop

Imagine, Design, Create – Cesar Rodriguez (cesar,.rodriguez@med.fsu.edu)

- imagine: Write it down!
    - Cell based therapeutics is the third pillar of medical therapeutics (small molecule → proteins/antibiotics → cells)
    - If[molecule]>x THEM produce therapy (molecule→senser→procduct→activator→product)
    - Sense environment; produce effect (ex. Smell bread→salivate)
- Design: design specification→simulation modification
    - Apple omnigrapho
    - Microsoft visio
    - Cell modeller
    - Jupyter (anaconda package)
    - Neuvidiaflex
- Create: Make your prototype
    - Gen9 (DNA synthesis company)
    - Twist (DNA synthesis)
    - Cloudlab (do your experiments in a remote lab)

Policy & Practices in DIY Bio and iGEM – David Lloyd

- Core of HP: Society←→ Lab
    - Social sciences based questions
    - Follows a methodology
    - Demonstrates real world application
    - Meaningful impact on your labwork
    - Discover things you didn't know before
- Outreach $\neq$ P&P (outreach is put out; P&P is bring in)
- Read the judge's notebook on website COMMUNICATE! TARGET AUDIENCE!

    - Make it obvious how you fulfill the criteria
    - Make sure you differentiate P&P vs. outreach
- Be careful of survey design!
    - If you are doing a survey, make sure you make it statistically relevant
    - Good example: Gender Study (Paris Bettencourt 2013)

Mathematical Modelling for SynBio – Brian Ingals

- Physical vs. conceptual
- Mathematical modesl can be mechanistic (description) or predictive (make inferences/extrapolation)
- Mass action kinetics
    - Chemical rxn: X→P
    - Rate constant: k=[P]/[X]
    - Etc.
- Programs that can be used
    - COPASI
    - MATLAB
    - XPPAIIT
    - Mathematica
- Separation of time scales; phase plane analysis
    - Processes slow → treated as frozen in time
    - Processes fast → treated as occurring instantaneously

Wiki & Visual Design in iGEM – Patrick Wu

- Design vs. Art (not the same thing)
    - Design: communicate the same thing to everyone (USABILITY is key!)
    - Art: Can have different interpretations to different people
- Content, accessibility, visual hierarchy, grid layout to organize info
- Interpret data for your audience! Figure captions and descriptions
- Summary page for judges is good for check-boxing

May 16, 2016

- Decided on BioBasic as our peptide synthesis company and ordered all of the peptides
- Ordered full length BBI as well as KTI (as a backup peptide) from Sigma Aldrich
- Wait for ~3 weeks for peptides to come!

June 6, 2016

- Dr. Goodarzi had suggested the idea of reducing our peptide; in the original structure of BBI, our truncated section contains 2 cysteines (which means the possibility of cysteine bridges) (Voss, R.-H., Ermler, U., Essen, L.-O., Wenzl, G., Kim, Y.-M. and Flecker, P. (1996), Crystal Structure of the Bifunctional Soybean Bowman-Birk Inhibitor at 0.28-nm Resolution. European Journal of Biochemistry, 242: 122–131.doi:10.1111/j.1432-1033.1996.0122r.x)
- Literature supports that reduction of our peptide would not affect its radioprotective effects (Gueven N, Dittmann K, Mayer C, Rodemann HP. The radioprotective potential of the Bowman-Birk protease inhibitor is independent of its secondary structure. Cancer Letters. 1998;125:77–82.)
- We chose to use DTT as our reducing agent from literature search

June 10, 2016

Clonogenics 0.1 – cell splitting protocol

- take HTC116 cell line with >50% confluency
- wash cell line twice with 1mL of DMSO
- add 1mL 37C trypsin and incubate for 4 minutes
- add 4mL of media (McCoy's with 10%FBS and 0.5%pen-strep) to each of 4 new plates

- add 3mL media to plate from step 3 to quench trypsin, pipette up and down to separate the cells (ideally we have no clumps in the cells but have them individually suspended in solution
- add 1mL of the cells from step 5 to each plate done in step 4, making sure to seed the cells all over the plate
- incubate at 37C with 5% CO2 to use for next time (over the weekend)

June 13, 2016

Clonogenic 0.1 – solvation of peptide

- dissolved our heptamer peptide (ALSYPAQ) in McCoy's Media, our peptide was not visible to the naked eye after adding McCoy's Media, so it's soluble(?). We then did a serial dilution with the peptide solution to make varying concentrations

concentration	volume of peptide solution	volume of media	total volume
1200μM	dry peptide	10mL	10mL
150μM	1mL from 1200μM	7mL	8mL
100μM	4mL from 150μM	2mL	6mL
50μM	2mL from 100μM	2mL	4mL

We got scolded...
** Remember for next time/actual experiment: only 2-10μL of drug/peptide treatment (negligable amounts) should be added to cell lines to do experiment instead of incorporating the peptide into our media

Clonogenic 0.1 – Cell counting and seeding protocol

- a ~50% confluent cell line of HCT116 cells
- removed media, washed twice with 25°C DPBS
- add 1mL of 37°C trypsin, incubated for 6min in 37C, 5% CO2 incubator
- quench effects of trypsin by adding 3mL media (McCoy's Media with 10% FBS and Pen-Strep) pipetted up and down to suspend cells
- put 4mL of solution containing cells into autoclaved 15mL falcon tube and centrifuged at 2500rpm for 5min at 25C
- removed all media, leaving only pellet of cell
- resuspended cells with 1mL media (removed 50μL to look at under the microscope to make sure clumping of cells is minimal)
- pipette 10μL of cell mixture and mixed with 10μL of typan blue; pipetted onto cell counting slide (10μL in each of well A and B)
- put the slide in cell counter:
    - Well A: live cells 9.15×10^5 cells/mL (69% live cells)
    - Well B: live cells 7.04×10^5 cells/mL (81% live cells)

Clonogenic 0.1

- The discrepancy between the two wells is quite big, meaning that we did not mix the cell mixture well before counting. Ideally we would want to work with cell cultures that are 85-100% live cells, so we should be careful of cell death (leaving trypsin on for less time, more cogniscent of bubbles etc) in coming experiments.
- But for the sake of practice we decided to seed the cells from the cell counting experiment (average of well A&B: 809500 cells/mL) by plating 6 plates of each of the following cell counts:

cell count/mL	cell solution volume	media volume	total volume
2000	37μL from 809500cell/mL solution	15mL	~15mL
1000	6mL from 2000cell/mL solution	6mL	12mL
500	4mL from 1000cell/mL solution	4mL	8mL

- 1mL of each solution was added to 4mL media on the plates. (6 plates for each cell count)
- the plates were incubated at 37C and 5% CO2 (will check up on them Friday) OUR CANCER BABIES!!!

June 14, 2016

Clonogenic 1.0

- BBI Treatment of HCT116 Cells - 7mer (ALSYPAQ)
    - Time of Application: 10:55 AM
    - Time of Irradiation: 4:10PM

Plates were treated with BBI by Sid (see put peptide in solution entry from Monday 6/13)

Plate #	Cell Density	BBI Concentration	IR Dosage
1-6	500 cells	0	0 Gy
1-3	1000 cells	10 microMolar	5 Gy
4-6	1000 Cells	30 microMolar	5 Gy
1-6	2000 cells	0	5 Gy

June 15, 2016

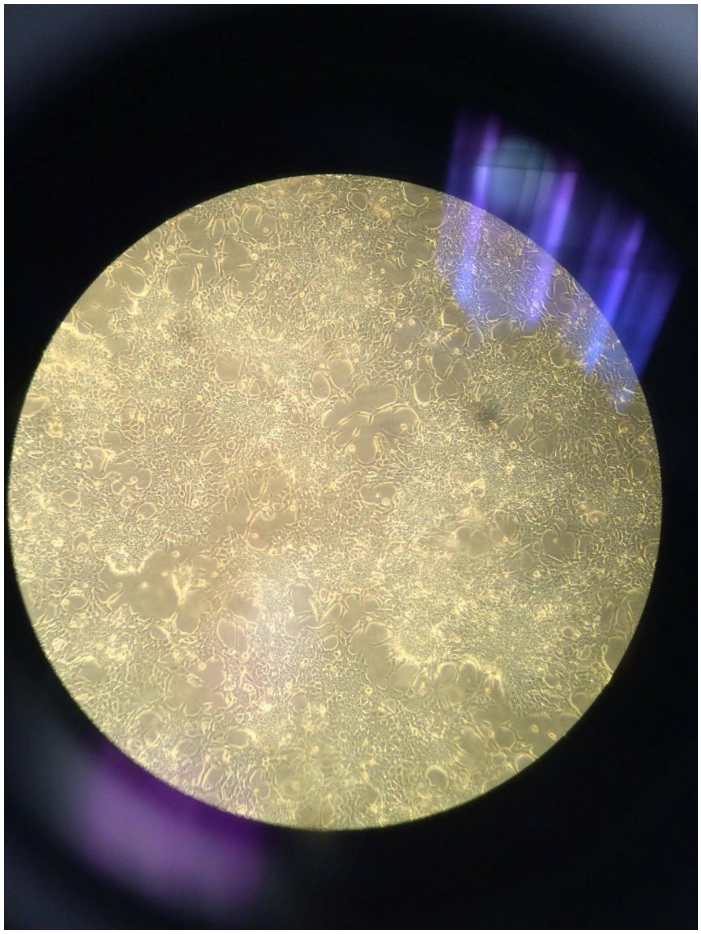
Clonogenic 0.1

- Made 500mL fix solution (3% acetic acid, 8% methanol, 89% water)
- stain solution (0.2% crystal violet, 10% formalin in PBS)
    - could not find formalin to make stain solution, we will get some from Nick Jette, but in the meantime we ordered some

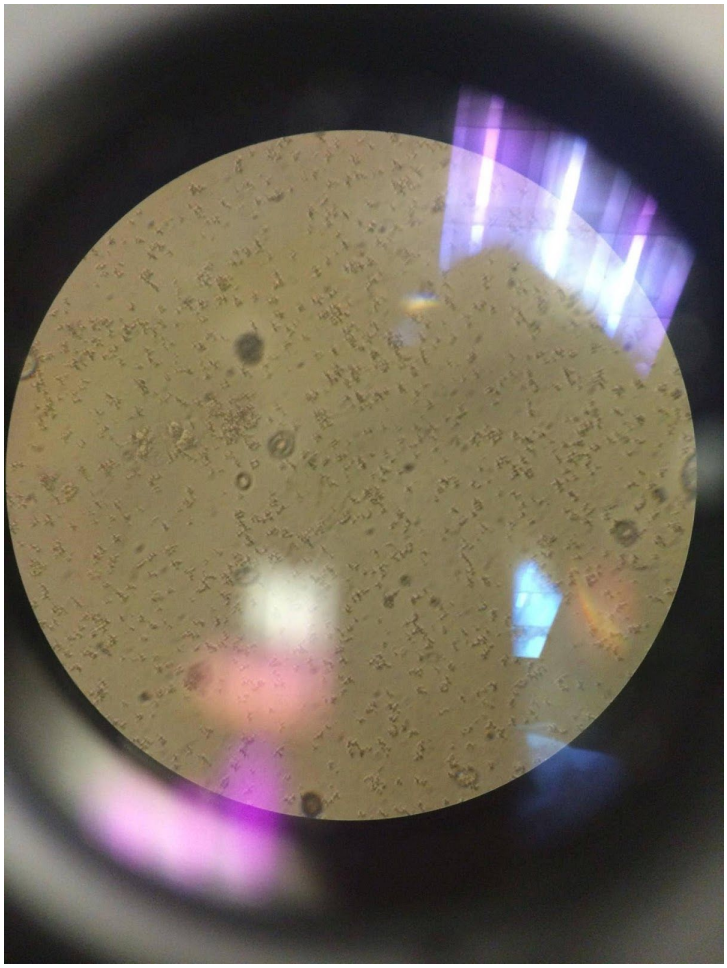
June 16, 2016

Clonogenic 0.1

- Some of our stock cells look very overgrown, so we split some of the less confluent ones, and we are going to throw away the ones that are more overgrown



- Looked at the cells we decided to irradiate and treat. The 1000 plates (treated with BBI and irradiation) looked very strange under the microscope as it had little specks that were not our cells. We suspect it is because our peptides were not soluble in McCoys media and clumped up in solution. So we will try to dissolve the other peptides in DMSO first before applying to our cell cultures. In the meantime, we are trying to salvage our heptamer by centrifuging to see if the peptides will form a pellet so that we can test a few more things on it before we toss it out.



June 17, 2016

Clonogenic 0.1 – Fixing and Staining protocol

- Our staining solution (06/15 entry) was not up to par to the standards as it did not look dark enough. The suspicion is that our crystal violet was not a the right concentration to begin with (as our crystal violet came in solution form instead of powder form), so we will go back and search up the initial concentration of our crystal violet. In the mean time, this practice will be done with crystal violet provided to us by the Susan-Lees Millers lab.
- Media was removed from plates
- 1mL fix solution was added (see entry on June 15); let sit for 2 min, then removed
- 1mL stain solution was added (see entry on June 15); let sit for 5 min, then removed
- deionized water was added to each plate (covers surface of the plate) to rinse the cells, removed

Clonogenic 0.1

- looked at stained colonies
    - the 500 plates had more colonies than the other 2 types of plates
    - 1000 plates had very little to no colonies
    - 2000 plates had about the same amount of colonies than 500 plates, but the colony sizes are much smaller

June 20, 2016

Clonogenic 0.2

- before we start to do our official trials, we needed to do another practice to familiarize ourselves with techniques
    - Learned that the P1000 pipettes were very effective in breaking up cells
- counted and seeded 48 plates with varying cell counts (x6 plates for each manipulation)

Drug (DMSO) Treatment Volume	0Gy Radiation	5Gy Radiation
0µL	sd. 500	sd. 2000
3µL	sd. 500	sd. 1000
6µL	sd. 500	sd. 1000
9µL	sd. 500	sd. 1000

June 21, 2016

Clonogenic 0.2

- Treated cells with varying volumes (Table15) of filter-sterilized DMSO as a carrier control as well as irradiated all our plates.
- Now to just wait for our babies to grow in the incubator!

Clonogenic 0.2 - For Thursday (10AM)

- More cell count and seeding practice
- counting practice plates from last friday in the cell colony counter and doing statistics on them
- setting up the vacuum media aspirator (maybe)

June 23, 2016

Clonogenic 0.1 - Colony Counting Protocol

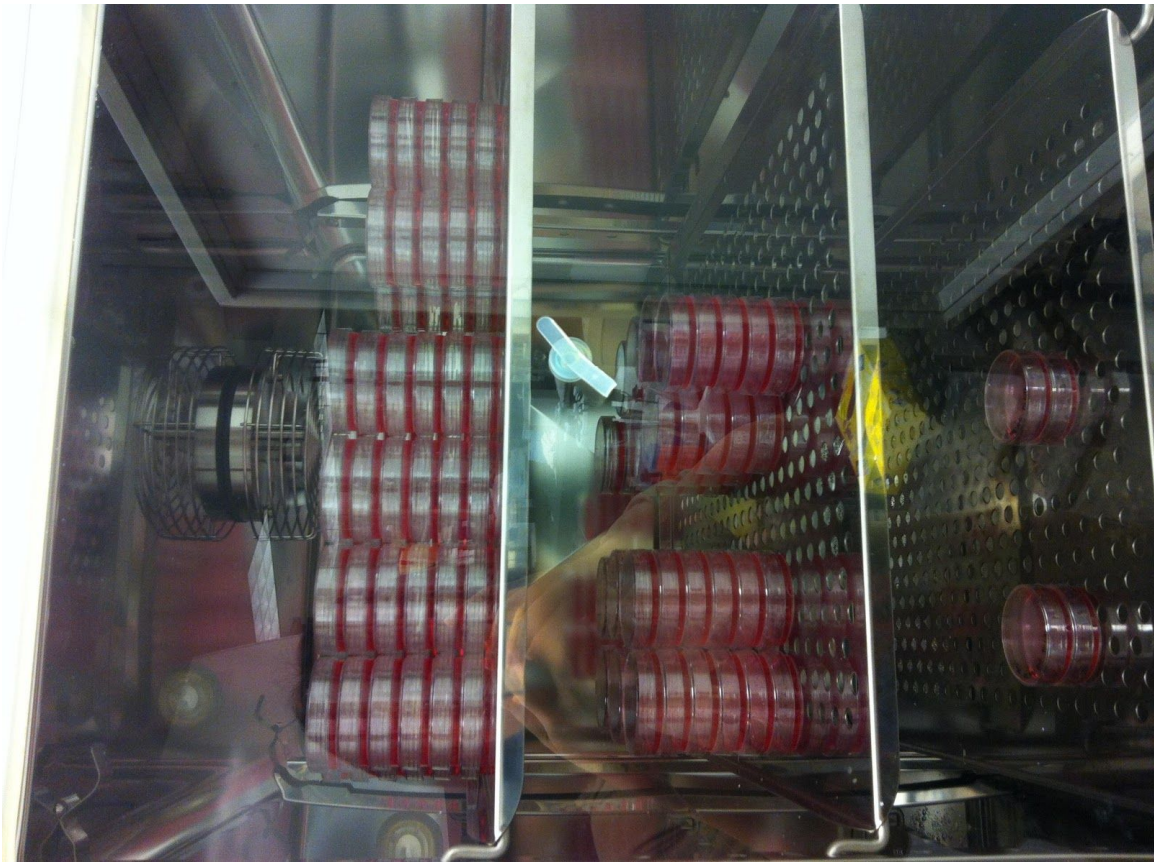
- Open the computer, select Colcount and the password is dnapk*atm
- Once on the desktop screen select colcount from the desktop
- Once the application opens click start, select 50mm UpperPosition (7E2) and then click Load
- Select Cell present and hit browse - use either Nick(good) or small colonies
- Insert the blank with no tip and it will start warming up.
- Optional - Save at desktop and make new folder every day
- Grab the plates with colony and start counting
- **Happy Counting**

Plates	500	1000	2000
1	46	9	22
2	84	1	10
3	63	12	22
4	53	13	8
5	42	7	19
6	43	7	8

June 27, 2016

Clonogenic 0.2

- Cells were trypsinized and counted using the Bio-Rad counter and plates.
    - Well A: 1.17 x 10^7 cells/ml (76% alive)
- 36 x 1000 cell plates and 36 x 500 cell plates were seeded with their respective amount of cells. 1ml of cell culture was added to 4 ml of McCoy's media with FBS and Pen-Strep.



June 29, 2016

HCT116 Clonogenics 1.0

- Our phosphorylated BBI peptide as well as our control scramble peptide came in!
- We tried to seed our 168 plates today (see google docs clonogenics trial 1), but in our cell count, it turns out only 12-14% of our cells are alive??! (even though we redid it 3 times)
    - we suspect that the cell counter was acting up as non of the other people's samples were working either. But either way those results are unusable, so we will have to push this off till next Monday to seed as this Friday will be Canada Day.

July 4, 2016

HCT116 Clonogenics 1.0

- Used stock cell plate with cells split on June 29th, also tried counting with cells from 2 weeks ago with 1000 seed but decided not to use them because cell survival was 9%
- Cell survival percentage of the stock solution used is average of 53%, decided to go ahead with the trials as we suspect it is our cell counting protocol that needed tweaking as opposed to our cells actually being fifty percent dead
    - Seeded 100 plates with 500 cells/plate
    - seeded 100 plates with 1000 cells/plate

July 5, 2016

HCT116 Clonogenics 1.0

- Plan of our clonogenics trial

	plates #	Volume added (uL)	Radiation(Gy)	Seeding	# Plates	Time drugged	Time irradiated
Control (DMSO+DTT)	1	0	0	500	3	11:20	
	2	3	0	500	3	11:21	
	3	6	0	500	3	11:25	
	4	9	0	500	3	11:28	
	5	0	5	1000	3	11:20	5:52
	6	3	5	1000	3	11:21	5:32
	7	6	5	1000	3	11:25	5:42
	8	9	5	1000	3	11:28	5:42
9mer (S)	1	0	0	500	3	11:30	
	2	3	0	500	3	11:32	
	3	6	0	500	3	11:35	
	4	9	0	500	3	11:40	
	5	0	5	1000	3	11:30	6:21
	6	3	5	1000	3	11:32	6:02
	7	6	5	1000	3	11:35	6:25
	8	9	5	1000	3	11:40	6:05
KSCl BBI	1	0	0	500	3	11:45	
	2	3	0	500	3	11:46	
	3	6	0	500	3	11:48	
	4	9	0	500	3	11:50	
	5	0	5	1000	3	11:45	5:52
	6	3	5	1000	3	11:46	5:36
	7	6	5	1000	3	11:48	6:02
	8	9	5	1000	3	11:50	5:36
Big BBI	1	0	0	500	3	12:00	
	2	9	0	500	3	12:02	
	3	18	0	500	3	12:05	
	4	27	0	500	3	12:08	
	5	0	5	1000	3	12:00	5:25
	6	9	5	1000	3	12:02	5:19
	7	18	5	1000	3	12:05	5:25
	8	27	5	1000	3	12:08	5:19
KTI	1	0	0	500	3		
	2	12	0	500	3	11:09	
	3	24	0	500	3	11:13	
	4	36	0	500	3	11:14	
	5	0	5	1000	3		6:21
	6	12	5	1000	3	11:23	6:17
	7	24	5	1000	3	11:21	5:05
	8	36	5	1000	3	11:20	6:13
Scramble	1	0	0	500	3		
	2	3	0	500	3	11:29	

	3	6	0	500	3	11:32	
	4	9	0	500	3	11:33	
	5	0	5	1000	3		5:56
	6	3	5	1000	3	11:37	6:20
	7	6	5	1000	3	11:40	6:27
	8	9	5	1000	3	11:41	5:47
P-Tyrosine 9mer (S)	1	0	0	500	3		
	2	3	0	500	3	11:52	
	3	6	0	500	3	11:50	
	4	9	0	500	3	11:48	
	5	0	5	1000	3		5:56
	6	3	5	1000	3	11:55	6:13
	7	6	5	1000	3	11:57	6:10
	8	9	5	1000	3	11:59	5:47

July 11, 2016

H2AX Protocol

- The cells are taken from 80-100% confluent flask and then are plated into desired number of glass slides.
- The plates are incubated in order to reach 100% confluency.
- Once the cells have reached 100% confluency they are divided into different treatment groups.
- The cells are treated with their respective treatments and following treatment they are washed with PBS at a certain period of time. (Note: The washes are poured onto the side, not directly on top of the slide.)
- Once the cells are washed, 100uL of fixative solution (3% w/v Paraformaldehyde, 2% w/v sucrose, 1X PBS). The cells are fixed for 10 min at room temperature.
- The cells are then washed with PBS twice after the fixing them.
- The cells are then stored for next day for antibody staining.
- Fixed, washed cells are then permeabilized by adding 100uL of Triton X-100 on the coverslip.
- Cells are then washed twice with PBS.
- Fixed and Permeabilized cells are then incubated with 100uL of primary antibody (H2AX, 53BP1 and H3S10p) diluted in PBS + 2% (w/v) BSA for 1 hour at room temperature.
- Cells are then washed three times with PBS and then incubated with 100uL of secondary antibody (Alexa-488, Cy3, DAPI) diluted in PBS + 2% (w/v) BSA for 20 min at room temperature in dark.
- Cells are then incubated with ~2mL of 4',6-diamidino-2-phenylindole (DAPI) diluted in PBS for 5-10 min at room temperature.
- Coverslips are then taken out and mounted onto the slides using Vectashield mounting media. The coverslip is then sealed with nail polish to keep it fixed at its position.
- Once the slides are ready, they are taken to the Axiovert microscope to count the number of foci.

July 12, 2016

HCT116 Clonogenics 1.0

- the cultures with p(y) 9mer looks more yellow than other plates, perhaps indicating more colonies (more to come in 2 weeks)

July 15, 2016

HCT116 Clonogenics 1.0

- We had set up a vacuum to suck off the media as well as treat our plates with fix and stain solution. Colonies were counted using ColCount colony counter.

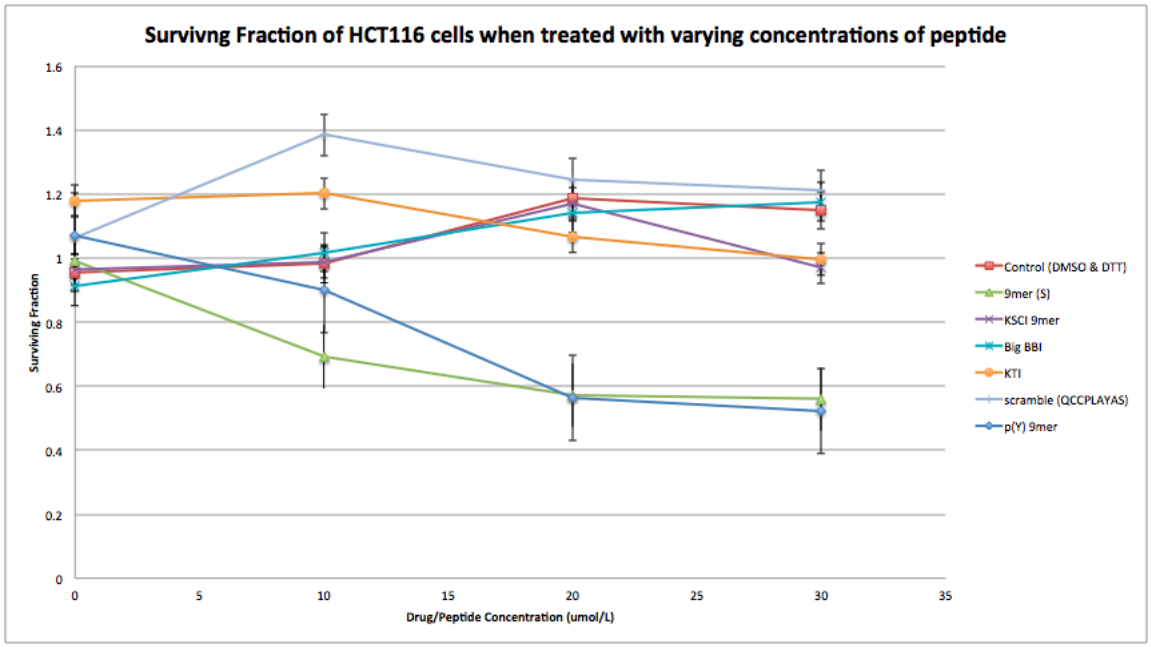
	Concentration (µmol/L)	Radiation(Gy)	Seeding	Colony Count			Treatment Concentration	Surviving Fraction	Trial PE
Control (DMSO+DTT)	0	0	500	250	200	168	0	0.955085482	**0.431375**
	10	0	500	178	242	215	10	0.98135806	
	20	0	500	237	311	220	20	1.186902347	
	30	0	500	223	270	250	30	1.148266203	
	0	5	1000	16	24	15	0	0.042499759	
	10	5	1000	37*	18		10	0.041727036	
	20	5	1000	31*	8	16	20	0.027818024	
	30	5	1000	20	28	6	30	0.041727036	
9mer (S)	0	0	500	208	220		0	0.992176181	
	10	0	500	77	191	180	10	0.692359703	
	20	0	500	122	129	120	20	0.573360379	
	30	0	500	106	117	140	30	0.560996813	
	0	5	1000	20	12	9	0	0.031681638	
	10	5	1000	17	30	27	10	0.057181493	
	20	5	1000	22	7	3	20	0.024727132	
	30	5	1000	13	9	26	30	0.037090698	
KSCl BBI	0	0	500	194	221	209	0	0.964358157	
	10	0	500	212	209	218	10	0.987539844	
	20	0	500	247	268	242	20	1.169902444	
	30	0	500	227	201	199	30	0.968994494	
	0	5	1000	48*	66*	50*	0		
	10	5	1000	23	6	13	10	0.032454361	
	20	5	1000	5	17		20	0.025499855	
	30	5	1000	85*	11		30	0.025499855	
Big BBI	0	0	500	184	202	204	0	0.911813001	
	10	0	500	250	198	211	10	1.018448759	
	20	0	500	256	236		20	1.140538974	
	30	0	500	225	290	246	30	1.176084227	
	0	5	1000	19	48*	11	0	0.03477253	
	10	5	1000	22	15	28	10	0.050226987	
	20	5	1000	18	10	23	20	0.039408867	
	30	5	1000	50	21	28	30	0.076499565	
KTI	0	0	500	247	267	250	0	1.180720564	
	10	0	500	251	286	241	10	1.202356805	
	20	0	500	222	236	232	20	1.066357578	
	30	0	500	198	240	206	30	0.995267072	
	0	5	1000	22	43*		0	0.05099971	

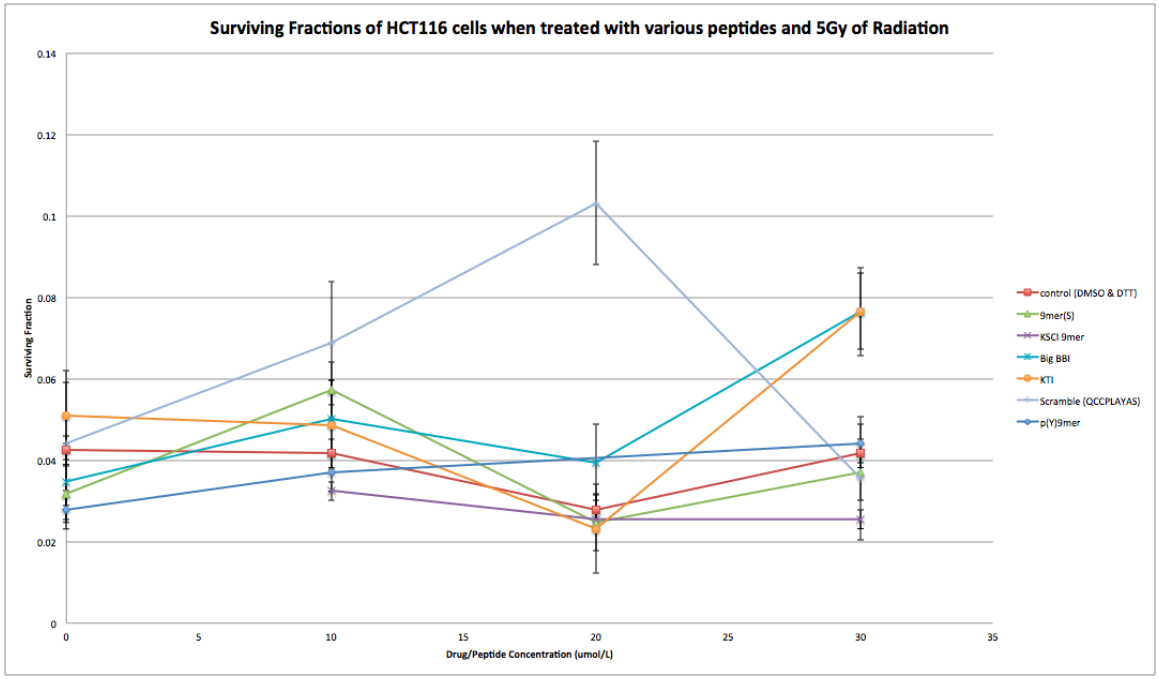
	10	5	1000	21	79*		10	0.048681542	
	20	5	1000	30	83*	33*	20	0.023181686	
	30	5	1000	27	30	42	30	0.076499565	
Scramble	0	0	500	218	227	243	0	1.063266686	
	10	0	500	278	283	335	10	1.384719405	
	20	0	500	271	284	252	20	1.247174732	
	30	0	500	248	295	240	30	1.210084034	
	0	5	1000	17	31	9	0	0.044045204	
	10	5	1000	25	33	31	10	0.068772337	
	20	5	1000	36	53		20	0.103158505	
	30	5	1000	21	13	12	30	0.035545253	
P-Tyrosine 9mer (S)	0	0	500	241	216	235	0	1.069448469	
	10	0	500	180	226	176	10	0.899449435	
	20	0	500	107	126	131	20	0.562542258	
	30	0	500	107	108	123	30	0.522360668	
	0	5	1000	10	14		0	0.027818024	
	10	5	1000	13	19		10	0.037090698	
	20	5	1000	51*	51*		20		
	30	5	1000	37	8	12	30	0.044045204	

>blank spaces indicates the plates were contaminated in the process of obtaining the data, as such data was not obtained and these plates were excluded from counting.

HCT116 Clonogenic 1.0 - Graphs

- Graph 1 is of plates seeded with just 500 cells and treated with just variuos peptides and no radiation to see if peptides inherently have toxicity to our cells. Data was created in excel with different treatments overlayed on top of one another. Error bars represent excel calculated standard error for those particular data points.
- Graph 2 is of plates seeded with 1000 cells and treated with various peptides as well as 5Gy radiation to see if our peptides confer radioprotection to our cells. Data was created in excel with different treatments overlayed on top of one another. Error bars represent excel calculated standard error for those particular data points. (note the scale difference between the two graphs)





July 18, 2016

<u>HCT116 Clonogenics 1.0 - Discussion</u>

- None of the peptides we intended to test showed significant radioprotection compared to out control with just DMSO and DTT (the solvent we dissolved our peptide in)
- BBI and KTI were interesting in that at 30µmol/L they seem to be conferring radioprotection to our cell-line compared to the control; but this relationship does not seem linear (no apparent linear correlation between application of the peptide and surviving fraction). Also cannot be sure of the significance of this observation due to the small increase that is apparent (being concious of the scale on the second graph)

- our scramble peptide seemed to increase the surviving fraction of irradiated cells <20μmol/L, but at 30μmol/L seemed to do nothing to help the surviving fraction of our cell-line. Only 2 datapoints were used in the calculation of the surviving fraction of cells treated with 20μmol/L scramble peptide so the error on this observation could be large--> requires more in-depth statistical analysis before conclusions.
- The Serine 9mer as well as the p(Y)9mer seemed to be killing our cells/making them unable to reproduce, suggesting toxicity against HCT116 cells or cancer cells in general; while the rest of our peptides were comparable to the control treatement in terms of toxicity (or lack thereof)
- KTI protein as well as our scramble peptide showed interesting results on cells that did not undergo irradiation, in that those peptides seem to increase the surviving fraction on our cell-line

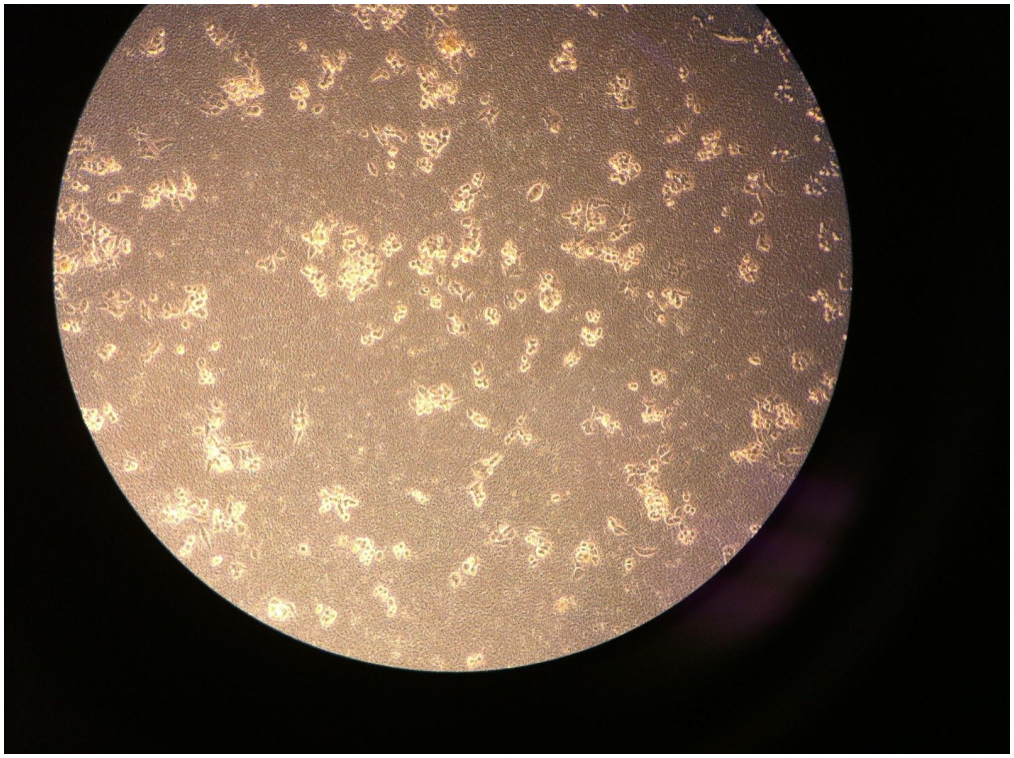
Next steps: Trypan Blue Assay Protocol

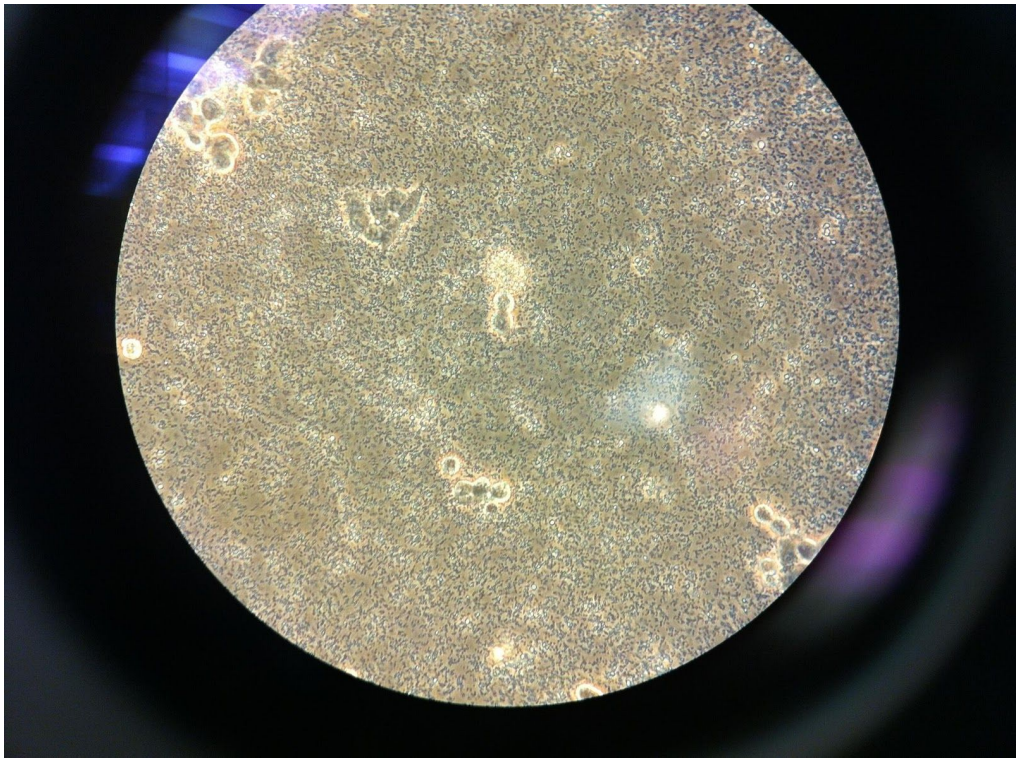
- Discussed next steps with our mentors and decided to do a Trypan Blue survivability assay in the interest of time. Trypan Blue Assay should be able to give us some analyzable results in the time-frame of a week
    - Grow, treat and irradiate the cell line as needed to test timing as well as different peptides (splitting cells without being concious of the number seeding)
    - Aspirate the media and place it in an autoclaved 15mL falcon tube
    - wash cells iwth DPBS and Trypsinize to lift cells off the plate, quench, then pipette the solution into the same 15mL falcon tube
    - Mix well and stain with trypan blue stain.
    - Quickly count total cells and live cell fraction with BioRad 500 cell counter. Record percentage of live cells and tabulate for analysis of survivability.

July 20, 2016

HCT116 Trypan Blue 1.1

- attempeted to count cells for trypan blue assay, but after trypsinizing for longer than usual (8min), the cells at the botom of the plate was unwilling to lift off the plate when viewed under a microscope. Seemed like there was a carpet of other smaller material around the cells that is trapping the cells to the bottom of the 60mm plate. Media was normal and was not cloudy (which is why we were unsure about what to think.
- Consulted this observation with our advisor, who announced that it was likely contamination. As a result all plates were discarded (all plates seemed to see the same situation) and the incubator was cleaned out as the water in the incubator was getting gross (which might have been the source of the contamination to begin with)
- Our HCT116 stock cells do not seem to be affected





July 26, 2016

H2AX Training 0.1

- Learned how to fix the cells

- Learned how to do antibody staining

July 27, 2016

H2AX Training 0.1

- Learned how to prepare slides with mounting media and nail polish

July 28-29, 2016

H2AX Training 0.1

- Learned how to set-up the microscope and use different filters

August 2, 2016

1BR3 Clonogenic 1.0 – Splitting protocol

- New Materials
    - Media was composed of MEM (no extraneous nutrients), 15% FBS, 1% Penstrep, 2mM L-Glutamine (Stock is 200mM or 100x)
    - used 0.25% trypsin
    - used DPBS with no additives

1. Fibroblasts were looked at under the microscope to ensure over 70% confluency
2. Aseptic technique practiced to a tee (turn on hood 15 min before using, warm up media and trypsin in the 37c water bath, spray and wipe down everything with ethanol, ensure we have sterile/autoclaved 15mL falcon tubes)
3. wash 2 times with DPBS (making sure to pipette PBS on the side of the flask away from our cells)
4. add 3ml trypsin in each flask, incubate at 37c for ~5min
5. bump side of flask to ensure all cells are rounded and deadhered (check under the microscope)
6. add media to quench trypsin effects (around 6mL)
7. pipette media around the flask to "wash" down the surface (making sure declumped and deadhered)
8. remove all media and place in a 15mL falcon tube
9. centrifuge tubes at 2500rpm, 4c, for 3 minutes; make sure there is a pellet (should be visible for a T75 flask)
10. while the tubes are being centrifuged, fill new flasks with 14-18mL of media (T75 flasks)
11. remove the falcon tubes from centrifuge, aspirate the media
12. resuspend cells in 6mL media (for a 1:3 split) or 8mL media (1:4 split)
13. add 2mL into each new flask
14. tilt back and forth for an even spread
15. place back into incubator and do not touch the cells again (to not disturb) until you need to use them next

H2AX Training 0.1

- Learned how to count foci
- Advised to use blind counting technique for actual experiment

August 5, 2016

1BR3 Clonogenic 1.1

- Trypsinized/ Lifted a T75 flask of 1BR3 feeder fibroblasts(passage #: 12; 80% confluency) and added to 10mL of media
- Irradiated cells at 35 Gy of radiation (^{134}Cs source, 85 Gy/min)
- Diluted cells to make 110mL of cell solution
- Seeded a layer of feeder cells on 108x60mm plates by pipetting 3mL of media and 1mL of seeder cells
- Placed in 37 ◦ C, 5% CO2 overnight

August 6, 2016

1BR3 Clonogenic 1.1

- Counted 1BR3 cells from our T25 flask stock
    - Well A: $5.98x10^5$ (52% live); Well B: $6.41x10^5$(48% live)
- Dilution and seeding of live 1BR3 cells to feeder plates

August 7, 2016

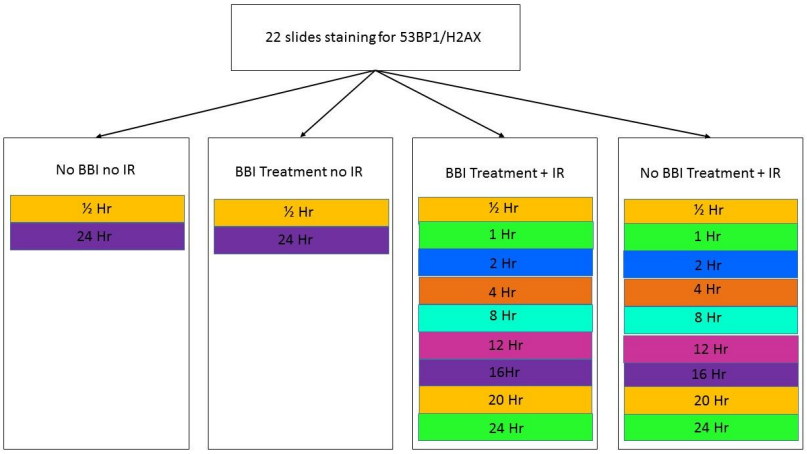
1BR3 Clonogenic 1.1

- Treatment with various peptides (big BBI, 9mer, p(Y) 9mer, KTI, DTT+DMSO, no treatment control)
    - Peptides were added to a final concentration of 20uM
- Irradiation with 3 Gy of radiation according to original plan and seeding
- Placed in Placed in 37 ◦ C, 5% CO2

August 8, 2016

H2AX Assay 1.1

- Cells were plated on glass slides (18) and after they reached 100% confluency they were put into different treatments



- The procedure was followed with 2Gy of radiation treatment
- Number of foci were counted twice using bling counting technique each time.

Treatment	Time	Count #1	Count #2	Average
BBI+, IR-	0.5	0.77	0.7	0.735
BBI-, IR-	0.5	1	0.82	0.91
BBI+, IR+	24	9.13	8.97	9.05
BBI-, IR-	2	21.13	20.7	20.915
BBI-, IR+	8	8.17	8.83	8.5

BBI-, IR-	24	6.06	6.13	6.1
BBI+, IR-	24	0.88	1.36	1.12
BBI+, IR+	2	19.59	16.7	18.145
BBI-, IR+	24	3.82	2.59	3.205
BBI+, IR+	8	7.41	6.24	6.825
BBI-, IR+	4	11.18	12.3	11.74
BBI-, IR+	0.5	29.5	32.03	30.77
BBI-, IR+	16	3.6	2.77	3.19
BBI+, IR+	16	1.15	1.37	1.26
BBI+, IR+	0.5	29.6	26.7	28.15
BBI+, IR+	4	12.7	8.1	10.4
BBI+, IR+	1	29.73	28.4	29.07
BBI-, IR+	1	32.1	30.8	31.45

August 11, 2016

1BR3 Clonogenic 1.1 – Contamination

- Found our stock flasks are contaminated
- Cleaned out our incubator and streaked reagents (media, DPBS, contaminated plate) on some LB plates to see what is causing the contamination
- Obtained more cells from Dr. Goodarzi – will be able to receive them in the near future

August 12, 2016

1BR3 Colonogenic 1.1 – Contamination results

- Non of our reagents were found to be contaminated through streaking on LB
- Thought about the possibility of our trypsin being contaminated but eliminated the possibility as trypsin is a protease and cell should not grow there
- Contamination most likely due to incubator not being clean, so we thermal cycled our original incubator, and in the meantime we have switched to using a different incubator to avoid further contamination

August 15, 2016

Big Team meeting

- Presented biotarget results to the whole team and all of our advisors
- Dr. Goodarzi, after seeing our presentation, gave us some suggestions on what to do going forward
    - Suggested using lower Gy (ex. 0.25, 0.5, 1.0) to test for hyper-radiosensitivity that is observed at low Gy → more applicable to project as typically astronauts receive radiation in 0.25 Gy range (Short S.C., Woodcock M., Marples B., 2003. Joiner M.C. Effects of cell

cycle phase on low-dose hyper-radiosensitivity International Journal of Radiation Biology, 79 (2) , pp. 99-105.)

- Suggested altering the concentration of peptide added to our H2AX assay to see the effects of altering concentration on DSB repair
- Suggested looking at more timepoints after radiation for H2AX assay to get a better trend of looking at when BBI works on our cells
- Will be providing more 1BR3 cells for us to work with; Interested in looking at a comparison of how our peptides work in cancer cells vs. primary fibroblasts

August 18,2016

1BR3 Clonogenic 1.2

- Trypsinized/ Lifted a T125 flask of 1BR3 feeder fibroblasts(passage #: 12; 80% confluency) and added to 10mL of media
- Irradiated cells at 35 Gy of radiation (^{134}Cs source, 85 Gy/min)
- Diluted cells to make 225mL of cell solution
- Seeded a layer of feeder cells on 220x60mm plates by pipetting 3mL of media and 1mL of seeder cells
- Placed in 37 ◦ C, 5% CO2 overnight

August 19, 2016

- Found a patent on a product that is very similar to ours with the inventors Minnie McMillan and Lynn E. Foster (Patent: http://www.google.ch/patents/US20150050250)

1BR3 Clonogenic 1.2

- Counted 1BR3 cells from our T25 flask stock
  - Well A: 4.02x10^5 (100% live); Well B: 2.92x10^5(100% live)
- Dilution and seeding of live 1BR3 cells to feeder plates
  - To make 115 mL cell concentration of 700 cells/mL for our 4 Gy radiation plates

$$\bar{x} = \frac{4.02\times10^5+2.92\times10^5}{2} = 3.47\times10^5$$

$$(347000\tfrac{cells}{mL})(x\ mL) = (700\tfrac{cells}{mL})(115\ mL)$$
$$x = 232\mu L$$

Radiation (Gy)	Seeding concentration (Cells/mL)	Volume for Serial Dilution		
		Vol from previous concentration (mL)	Vol Media (mL)	Total Volume (mL)
4	700			115
2	500	75	30	105
1	250	65	60	125
0.5	200	85	21	106
0.25	175	66	9	75
0	150	35	5	40

- Plates previously seeded with feeder layer (August 18) were plated 1mL of cells diluted to the specified concentrations in the table above according to intended radiation treatment
- Plates treated with various types of peptides (big BBI, 9mer, p(Y) 9mer, KTI, DTT+DMSO, control) (4-5PM)

August 20, 2016

1BR3 Clonogenic 1.2

- Cells irradiated with 137Cs source (8.5Gy/min) and designated Gy (0.25/1.7s, 0.5/3.5s, 1/7s, 2/14.1s, 4/28s) at 8AM (total incubation with peptides before radiation: 17hr)

August 22, 2016

1BR3 Clonogenic 1.2 - Contamination

- Plates for both the Clonogenic assay and the H2AX assay were found to be contaminated when looked at under the microscope (40x magnification); both assays are not able to continue
- Contaminated plates were disposed of; kept a few plates for reference to test the source of contamination

August 23, 2016

1BR3 Clonogenic 1.2 - Contamination

- Informed Nick Jette regarding contamination, he suggested we stop working out of the refrigerator as our chassis team, which may be the root of our contamination
    - He will contact Dr. Goodarzi to see if we will be able to use some of his lab space so that we do not get any further contamination
- Look for the root of the contamination by plating various reagents on 60mm plates
    - New unopened MEM (control plate)
    - Old media (5mL)
    - DPBS (1mL) + MEM (4mL)
    - Trypsin (1mL) + MEM (4mL)
    - MEM (5mL) + assortment of mBBI peptides
    - MEM pipetted by Nick (to test technique)
    - MEM pipetted by Nilesh (to test technique)
    - MEM pipetted by Sid (to test technique)
    - MEM left open in the incubator (to test bacterial growth in incubator
- Plates were left in the incubator (37C, 5% CO_2) overnight

August 24, 2016

1BR3 Clonogenic 1.2 - Contamination Results

- No growth observed in plates containing new or old media, DPBS, media plated by all experimenters
- Bacterial growth found in plates containing trypsin (viewed under 100x)
- More cells (1 x T75 flask (50% confluent as of today)) will be obtained (we will most likely when our trypsin comes in)
- Wendy (lab technician) donated 5 x T75 flasks as well as 60 x T25 flasks to us

August 25, 2016

Oxidized vs Reduced BBI - Immunofluorescence (h2ax)

- 4 plates of 1BR3 cells were allowed to reach 100% confluency.
- Oxidized BBI at 30uM of concentration were administered to two plates, while the other two received reduced BBI.
- The BBI was reduced with DTT, and it was ensured that a concentration of 500uM or higher of DTT was maintained in the plate.
- Cells were then radiated at 2Gy, and fixed at 4hrs and 8hrs using PFA.

August 25, 2016

Oxidized vs Reduced BBI - Immunofluorescence (h2ax)

- Immunofluorescence protocol was followed for antibody staining.
- The coverslips were then mounted on slides and the number of foci were counted using axiovert microscope.

Time/BBI	Oxidized BBI	Reduced BBI
4hrs	10.96	10.3
8hrs	5.43	5.86

September 1, 2016

Dosage Study

- 20 Plates of 1BR3 cells were allowed to reach 100% confluency
- Non-reduced BBI at 30uM concentration was administered to cells 6 hrs prior to radiation.
- All the cells/plates were fixed at 15 min using PFA in order to examine the quenching abilities of BBI.

September 2, 2016

<u>1BR3 Clonogenic 2.0</u>

- Trypsinized/ Lifted a T75 flask of 1BR3 feeder fibroblasts(passage #: 12; 80% confluency) and added to 10mL of media
- Irradiated cells at 35 Gy of radiation (^{134}Cs source, 85 Gy/min)
- Diluted cells to make 110mL of cell solution
- Seeded a layer of feeder cells on 108x60mm plates by pipetting 3mL of media and 1mL of seeder cells
- Placed in 37 ◦ C, 5% CO2 overnight

Dosage Study

- Cells were then stained using a primary antibody 53BP1 (The shame)
- The coverslips were then mounted on the slides and the number of foci were counted using axiovert microscope.

BBI/Gy	0	0.5	1	2
0	0.72	3.63	17.2	13.33
10	24.2	6.875	21.43	16.43
30	1.57	15.97	28.73	25.5
60	18.83	13.03	16.47	0.677
90	2.3	1.472	26.76	16.19

September 3, 2016

1BR3 Clonogenic 2.0

- Counted 1BR3 cells from our T25 flask stock
    - Well A: 2.56x10^5 (93% live); Well B: 2.01x10^5(98% live)
- Dilution and seeding of live 1BR3 cells to feeder plates
    - To make 115 mL cell concentration of 700 cells/mL for our 4 Gy radiation plates

$$\bar{x} = \frac{2.56\times10^5+2.01\times10^5}{2} = 2.28\times10^5$$

$$(228000\frac{cells}{mL})(x\ mL) = (700\frac{cells}{mL})(57\ mL)$$
$$x = 175\mu L$$

Radiation (Gy)	Seeding concentration (Cells/mL)	Volume for Serial Dilution		
		Vol from previous concentration (mL)	Vol Media (mL)	Total Volume (mL)
4	700			57
2	500	37	15	52
1	250	32	31	63
0.5	200	43	10	53
0.25	175	33	4	37
0	150	17	3	20

- Plates previously seeded with feeder layer (September 2) were plated 1mL of cells diluted to the specified concentrations in the table above according to intended radiation treatment (4-5 PM)

September 5, 2016

1BR3 Clonogenic 2.0

- Treatment with various peptides (big BBI, 9mer, p(Y) 9mer, KTI, DTT+DMSO, no treatment control)
    - Peptides were added to a final concentration of 20uM
    - Done at 7 AM
- Irradiation with various Gy of radiation according to original plan and seeding
    - 1 PM (for total 6 hr. incubation with peptide)
- Placed in Placed in 37 ◦ C, 5% CO2

September 16, 2016

1BR3 Clonogenic 2.0

- Contamination found in all of clonogenic plates again. Will not have time to do another clonogenic trial
- Suspect the incubator being the problem as it was found that the air filter has not been changed on the incubator for a while
- setting up a collaboration with Goodarzi lab to use their incubator for future H2AX (will not have time to do clonogenics and deemed that if results are found of H2AX, it should be pretty conclusive)

September 17, 2016

Immunofluorescence Assay 1.2

- 22 plates of cell were allowed to reach 100% confluency
- The plates were administered non-reduced BBI at 30uM concentration 6hrs prior to radiation.

- Cells were irradiated and the fixing was done using PFA at regular intervals - 0.5, 1,2,4,8,12,16,20,24hrs. (Two more time points are added to the previous assay).
- Once the cells were fixed, antibody staining was done using 53BP1 primary antibody and secondary antibody (FITC and Cy3) were used for visualization.

September 18, 2016

Immunofluorescence Assay 1.2

- Once the cells were fixed, antibody staining was done using 53BP1 primary antibody and secondary antibody (FITC and Cy3) were used for visualization.
- The coverslips were then mounted on the slides.

September 29, 2016

Immunofluorescence Assay 1.2

- The number of foci were counted using axiovert microscope.

A	B	C	D	E	F
0	Time (Hr)	BBI-/IR-	BBI+/IR-	BBI+/IR+	BBI-/IR+
1	0.5	0.58	0.37	38.4	40.1
2	1			34.7	39.65
3	2			28.85	32.55
4	4			25.27	27.95
5	8			14.57	17.48
6	12			5.57	8.22
7	16			4.9	5.85
8	20			1.74	2.65
9	24	0.8	0.72	1.66	2.43

Chassis Journal

May 3
Our first day in the Lab. We started off by taking an inventory of reagents and supplies to figure out what we had and what we needed to order. Organization and inventory of the fridges and freezes also started but the -20℃ freezer needed to thaw after having sat for two years. A number of items were sent down to autoclave to ensure that we have materials ready for work. Lastly, agar plates with antibiotics were made following the protocol for LB Media.

May 4
We continued to work on the organizing the -20 once it thawed. We also started to tackle the fridge. There were several dozen plates that had been sitting in the fridge, drying out, over the last two years. The biggest problem was dealing with poorly labelled tubes or tubes that had no label. When in doubt, throw it out!

After looking through the -80 freezer, Dan found some *E. coli* cells (3073C and TOP10) and we used these to test our plates. There were both competent *E. coli* and *E. coli* with chloramphenicol (Chlor) resistance. Cells were spread onto each type of plate (Chlor and Amp) and into liquid media (Chlor)

May 5
Unfortunately, the Chlor resistant *E. coli* were able to grow on the Amp plates, there was a lawn of red cells. Indicating that the concentration of ampicillin (Amp.) was not great enough. There may have been some contamination of the Amp plates, as odd yellow growths were observed.

Organization of the freezer continued. There was a lot in there…

Overnight cultures were made of TOP10 cells and C3037 cells. Two 5mL cultures were made of each bacteria. They were incubated at 37℃ and 200rpm

Solutions for making cells competent were made: 50mM $CaCl_2$ (5mL 1M $CaCl_c$ and 95mL of ddH_2O) & 50mM $CaCl_2$ with 15% glycerol (5mL 1M $CaCl_2$, 15mL Glycerol, 80mL ddH_2O)

May 6
Made competent cells following the competent cell protocol. Four subcultures were made, two for each of the TOP10 and C3037. It took approximately four hours to grow the cells to the correct density necessary for competency. The competent cells were stored at -80℃.

May 9
We had a big group brainstorming session to identify goals and assays for the summer. The chassis came up with four goals: Transform and integrate our constructs into *Bacillus*; find the optimal media for cell growth in a patch; have sustainable expression of BBI; have BBI secreted by *Bacillus*. In general, the main experiments that we wanted to focus on are growth curves and cloning.
We used the competency test kit from igem to see how competent our cells were. 100ul aliquots of TOP10 and C3037 were transformed with the pSB1C3+RFP at the following concentrations: 0.5pg/ul, 5pg/ul, 10pg/ul, 20pg/ul, 50pg/ul. Transformation occurred following the transformation protocol.

May 10
Of the 10 aliquots transformed with pSB1C3 containing RFP, there were three Chlor plates that contained growth. Two plates that grew had been transformed with 5 pg/ul and the third plate was a concentration of 0.5 pg/ul. All of the bacteria that were transformed were *E. coli* TOP10. There was a lawn of growth of clear and colourless bacteria on both the Chlor and Amp plates. The Amp plates were for the negative control. The strange growths are a sign of contamination and that the concentration of Amp is too low.

A colony PCR was run to check that the plasmids we hopped to transform into *E. coli* were actually there. There was an issue with the strip tubes, apparently the lids had not been closed all the way. Resulting in evaporation of the liquid in all but 4 of the tubes. The last step was to confirm the PCR with a agarose gel. A 1% agarose gel was prepared according to the gel electrophoresis protocol. 2 lanes were run with 1KB plus DNA ladder from AMRESCO.

Two overnight cultures of 5 ml each were generated from each of the transformed plates. The tube were incubate at 37℃ with shaking at 200rpm.

New Amp plates were poured but with a greater concentration of Amp, 100 ug/mL.

May 11
cPCR was repeated but with colonies with from the two master plates made from the *E. coli* TOP10+RFP. Twelve samples total used.
The following conditions were used: 3 min @ 95℃; 30 sec @ 95℃; 30 sec @ 62℃; 30 sec @ 72℃ and repeat the last three steps 33 times; 7 minutes at 72℃. The cPCR was then confirmed with a gel. Unfortunately, there did not seem to be amplification of the RFP gene. There were no bands present on the gel.

In the meantime, glycerol stocks of the *E. coli* and the pSB1C3+RFP were made following the glycerol stock protocol. Stocks were stored at both -20℃ and -80℃.

May 12
We reacquainted ourselves with the procedure for restriction digests and ligations. We chose two registry parts from the kit plates to work with.

Part	**Description**	**Kit**	**Plate**	**Well**
BBa_J23100	Constitutive promoter	2013	5	18C
BBa_E0240	RBS+GFP	2014	2	24B

The DNA from the Registry kit plates were rehydrated with 10µL of ddH_2O and let to sit for ten minutes at room temperature. We could tell that the DNA was being mixed due to the red solution in the well. We followed the rehydration protocol.

The two different parts were digested separately following the protocol Dan gave us for restriction digests. The promoter was digested with PstI and SpeI; RBS+GFP was digested PstI and XbaI. These enzymes were chosen as to create a scar between the different constructs. The scar removes any restriction site,

permanently binding the two constructs together. The reactions were incubated at 37℃ for an hour followed by twenty minutes at 82℃ and stored at -20℃

Outside of the wet lab, planning started on the genetic circuits. In the operon we wanted a strong promoter (pGrac), rbs, and a double terminator (BBa_0010).

May 14

The restriction digest products were ligated together following the ligation protocol from Dan. 7µL of vector and 5µL of inset were used with the appropriate volume of buffer, water, and ligase. The reaction was incubated at room temperature for one hour.

Following the ligation, the products were used to transform *E. coli* TOP10 following the transformation protocol. The *E. coli* was plated on both Chlor and Amp plates. Amp was used as the negative control and to test the viability of the Amp plates. The *E. coli* were incubated overnight at 37°C.

Overnight cultures of the *E. coli* TOP10 transformed with the pSB1C3+RFP from May 9th were made in LB media. The cultures were grown overnight at 37°C with shaking at 200rpm.

May 15

The transformation results were observed. There were no colonies on the Amp plates, a good sign that the new concentration of Amp was sufficient. There were colonies on the Chlor plates but there was no visible GFP in natural or U.V light.

Using the overnight cultures from yesterday, we familiarized ourselves with the plasmid extraction (miniprep) protocol. The plasmids were extracted using the E.Z.N.A Plasmid Mini Kit (Omega Bio-Tek) and following its protocol. Three sample of the pSB1C3 were minipreped. Plasmid yield was checked using nanodrop spectrophotometer at an absorbance of 260nm. The caveat with this method is that the genomic and plasmid DNA cannot be differentiated based of A_{260}. The yields were quite low: 1.7, 3.1, and 62.7ng/ul. The purity of the plasmids was also quite bad, according to the $A_{260/280}$ value.

We rechecked our Chlor plates to see if the concentration of Chlor was sufficient. 50ul of untransformed *E. coli* was plated onto a Chlor plate and incubated at 37°C overnight.

May 16

Today we took care of minor housekeeping issues, 500mL of LB broth and 1000mL of LB agar. These were made following the recipes for broth and agar. 0.45mL of 50mg/uL chloramphenicol was added 750mL of agar while 250mL of agar was utilized for plain plates.

cPCR practice continued with *E. coli* TOP10 transformed with pSB1C3+RFP. A 4.5 times master mix was made for the four colonies selected from the plates. Volume of master mix used was 17uL with a total reaction volume of 25uL. Prefix and suffix primers were used. The same PCR conditions were used from May 11th.

May 17

Repeated yesterday's cPCR but with four different colonies. Unfortunately, there were no bands on the gel that we ran to confirm the pcr. Why tried to troubleshoot our protocol and the best explanation was that the annealing temperature was too high, preventing the primers from binding.

We got new *E. coli* TOP10 cells from Deirdre and streaked the cells onto plain agar plates and incubated them at 37°C overnight.

May 18

There was a lawn of growth of was observed on the plate that was spread with the *E. coli* TOP10.

May 19

We found *B. subtilis* spores from the 2014 iGEM team. We tried to germinate the spores by rehydrating the spores in 200ul ddH_2O. 50 ul of the resuspended spores were plated on plain lb plates while another 50ul was added to two overnight cultures. Both the plates and culture tubes were left to incubate at 37℃.

New glycerol stocks of *E. coli* TOP10 were made from the single colonies that were isolated from the cells that Deirdre gave us. The stocks were made following the glycerol stock protocol and stored at -20 and -80℃.

Since our gel worked yesterday, we decided to gel extract the band. The band we extracted was found at 1200 base pairs. The band was extracted using the Omega-Biotek E.Z.N.A gel extraction kit and following its protocol. Since we wanted to send the extracted DNA down to get sequenced, we discovered that we could not elute in elution buffer. The sequencing people demand that the DNA is only in ddH_2O. When we did elute the DNA from the column the yields were quite low, 3-5ng/ul, according to the nanodrop. Following Rai's advice, we added either elution buffer or water to the column, 30ul, and heated the column at 70℃ for 15 minutes. We then respun the columns. The yield improved dramatically, 22-43.2ng/ul.
The extracted plasmids were then prepared for sequencing following the sequencing lab's instructions. In a 0.2ml tube the following were mixed:

- 50-100ng of DNA for every 1000bp
- 3.2pmol of primer
- ddH_2O to 12ul total volume.

It is important to include the total plasmid length when determining how much template is needed.

May 20

After about a 18 hours of incubation, there was no growth or germination from the spores. This really wasn't that surprising since we don't know what has been done to these spores over two years ago. We left them t incubate over the weekend just encase.

There was no growth on the Chlor plate that was streaked with the new *E. coli* cells that we got from Deirdre!

Apparently more testing of e. Coli and Chlor plates

May 24

The spores did not germinate over the weekend after being incubated at 37℃ and grown on LB media.
The untransformed *E. coli* did not grow on the Chlor plates, which is what we wanted. This means that there is no contamination of either the plates or the cells.
We took care of minor housekeeping items such as making more TAE buffer. We also started to look around the lab for any reagents that we might have to make various medias in the future.

June 1

It's june!

We found more spores from the 2014 team in the fridge and in what we suspect is water. We plated 50ul of the culture onto plain agar plates and incubated them overnight at 37℃.

June 6

A gram stain was done to see what state the *B. subtilis* spores were in. A gram stain was used as the spores would not stain and appear colourless under the microscope, while the vegetative cells stain purple (gram positive). Looking at the results of the stain under the microscope, there were primarily spores that had not been stained. There were some purple, rod shaped cells but they weren't very clear. The magnification used was 40x. Similar results were seen when cultures were taken from the culture tubes rather than the plates.

June 7

One liter of LB agar and 500mL of LB broth were made following the recipe for LB media. The broth is for growth curves that will start tomorrow. We ended up having to redo the LB plates as we think someone washed the bottle containing the media with soap :(. To prep for the growth curves 4 tubes of 1 mL LB-Chlor with a loop of *E. coli* TOP10 and incubated at 37.5℃ overnight.

June 8

We started the first growth curve. We are working with *E. coli* to practice and get a sense of how bacteria grows. One milliliter of cultured bacteria from the overnights was added to two flasks of 30mL of LB broth with chloramphenicol. One flask was kept at 4℃ as the negative control while the other was grown at 37.5℃. Growth was quantified by optical density, measured at 600 nanometers. Measurements were taken every half an hour. After each hour, 250 uL of each culture was plated onto Chlor plates with dilutions of 1:1000, 1:10000, and 1:100000.

June 9

We counted the cells that grew on our plates. We had so much counting that we enlisted everyone in lab! The 1:1000 dilutions contained too many cells to while the higher dilutions were reasonable. Based on the cell counts we realised that we needed to refine our approach to be sure we replicated the conditions in the patch as faithfully as possible.

More brainstorming was done to nail down our plan for growth curves. The biggest issue was that the OD values were above 0.4 for most of the experiment. Values over 0.4 are generally not considered accurate (for reasons). The next issue was how much to dilute the cultures when plating. To determine the best dilution, we made spot plates of the *E. coli* at dilutions from 1:10-1:1000000000.

Lastly we made LB plates doped with hygromycin to prepare for picking up *Bacillus subtilis* from Dr. Wong tomorrow.

June 10

Because our plates were not super dry, we had to leave the spot plates out overnight so that they could dry sufficiently before being placed in the incubator this morning.

To get a better idea of what temperature or patch will be at, we measured the different skin temperatures of everyone in the lab. Dr. Mayi was able to acquire a special thermometer from the U of C Medical Clinic. We found that the average skin temperature was around 35°C, which by a nice coincidence is the optimum temperature for *B. subtilis*. From this data, we decide to test growth at three temperatures: 4, 22, and 35°C. 22°C was selected as it is the average temperature of the International Space Station. This would be to test what would happen in if the spores germinated prematurely when in space.

We improved our plan for growth curves. We decided on four temperatures:

- 4°C negative control
- 22°C room temperature of ISS
- 35°C skin temperature
- 37°C positive control

We plan to inoculate 100mL of LB media with *E. coli* and every hour measure and spot growth and colonies respectively. With growth, we would be diluting the culture when the OD values climb above 0.4. Each run would take two days to do. The night before measurements start, media will be inoculated at 5:40pm and 12:20am. This is done in order to cover a 24 (48) hour time period within an 8 hour period.

Lastly, we thought of different ways we could test the viability of spores, the list was very extensive but it is a fairly low priority.

June 14

The *B. subtilis* spores germinated on the LB-Hygro plates! There was a band of growth around the filter paper. We then made an overnight culture of the freshly grown *B. subtilis* we incubated the *B. subtilis* at 30℃ instead of 37℃ as per Dr. Wong's instructions. An overnight culture of TOP10 was set up in chloramphenicol media and incubated at 37℃.

June 15

The streaked LB plate from yesterday was taken out of the incubator at precisely 9:30 am so that glycerol stocks of the *B. subtilis* could be made with the cells in log phase rather than stationary. Dr. Wong assured us that this would produce glycerol stocks with higher cell concentrations than our usual method. The glycerol solution was 250ul of plain LB with 250ul of 100% glycerol leaving us with 50% glycerol (v/v). Colonies were lifted from the plate using the streak loop. The new stocks were stored at -80℃. A new *B. sub* streak plate was made on hygromycin agar as the new master plate.

The TOP10 overnight culture was minipreped using the E.N.Z.A Plasmid mini kit from Omega. We followed the spin protocol with two modifications (1) columns were washed with 300uL of DNA wash in order to prep the columns and (2) prior to the elution step, the columns were heated at 70℃ for 15 minutes. This was done in the hope that it would boost the low yields we had been seeing. When the DNA was eluted, the yields were quite low, 4.4 and 5.2ng/uL respectively. Purity values were also poor: 260/280= 1.71 and 1.35; 260/230= 0.35 and 0.30.

We asked Deirdre what could be done to improve our persistently low yields. She told us two things: first was that we should have a longer lysis stage with more continuous inversion; second was to elute twice with 30uL of elution buffer after letting the buffer sit in the column.

New overnights of TOP10 started for more minipreps tomorrow.

June 16

The minipreps were done using the new Omega-Biotek plasmid mini kit that we ordered as well as the Sigma Aldrich Genelute that Deirdre lent to us. Protocols from each kit were followed.
The results are the following:

Sample	Concentration	A260/280

EZNA Omega Bio-Tek	27.5 ng/uL (in 100 uL)	1.78
Sigma Aldrich	37.2 ng/uL (in 50 uL)	1.84

DNA from IDT (*E. coli* BBI Construct) was rehydrated using the protocol found on their website:

1. Spin down for 3-5 seconds
2. 20ul ddH2o added to get a concentration of 50ng/ul (5x stock). The official protocol is to add enough water to reach a concentration of 10ng/ul. TE buffer was the recommended for rehydration as it is better for long term storage which is not a concern for us.
3. Tube was vortexed
4. Incubated for 20 minutes at 50℃
5. Store at -20℃

Both the extracted plasmid and rehydrated linear DNA were digested using EcoRI and PstI using the protocol from the 2014 Ucalgary iGEM team. The digests were incubated for 1 hour at 37℃ and then 20 minutes at 80℃. Following the digest, the products were ligated together using the T4 ligase from NEB, following the protocol again from 2014 Ucalgary iGEM team.

June 17

More DNA from IDT was rehydrated (BBI 5-GFP for *E. coli*) following the protocol used the previous day. 1.65ul of 50ng/uL of BBI-GFP and 1ul of 27.5ng/uL of pSB1C3+RFP were digested with EcoRI and PstI following the restriction digest protocol. Products of the digestion were ligated again following the ligation protocol.

The solutions for our new homemade miniprep kit were made:

- P1: 50mM Tris-HCl pH 8, 10mM EDTA, 100ug/mL RNase A
- P2: 200 mM NaOH, 1% SDS (V/V)
- P3: 3.0M potassium acetate pH 5.5

N.B: The EDTA will not dissolve in water until it is in a basic solution. It will then need to be pH corrected. The potassium acetate will also need to pH corrected as well.

June 20

Mostly Housekeeping things were done. New master plate of TOP10 was made on a plain agar plate and incubated at 37℃ overnight. We also tested glycerol stocks that we found were stored in -20℃ rather than -80℃. A loop of TOP10 stock was plated on plain lb and incubated overnight at 37℃. And to check whether the competent cells were still competent, they were transformed with BBI-GFP as a test. The transformed cells were plated on Chlor plates.

June 21

Not much happened today, more competent cells and glycerol stocks were made. The an aliquot of competent cells were used as a test batch. The aliquot was transformed with BBI-GFP in the pSB1C3 vector.

June 22

Start of the improved growth curve experiments. Three different 50mL falcon tubes with 9mL of hygromycin media were inoculated with 1mL of overnight *B. subtilis* culture at three different time points: 4:40pm,

12:20am and 8:00am. This was to cover 24 hours of growth in one work day. Growth was measured by a spectrophotometer at an optical density of 600nm. Each sample was measured every hour for eight hours. 100uL of culture was removed for measurement and diluted 1:10 in fresh media. This was to prevent the OD from climbing above 0.4. The results are as follows:

Time (h)	T=4C	T=22C	T=35C
0	0.029	0.01	0.011
1	0.034	0.036	0.028
2	0.006	0.029	0.043
3	0.016	0.008	0.059
4	0.009	0.01	0.12
5	0.005	0.014	0.171
6	0.002	0.02	0.196
7	0.014	0.036	0.217
8	0.04	0.047	0.308
9	0.031	0.089	0.293
10	0.009	0.084	0.292
11	0.017	0.084	0.374
12	0.005	0.1	0.392
13	0.001	0.093	0.395
14	0	0.108	0.427
15	0.009	0.12	0.394
16	0.029	0.148	0.384
17	0.029	0.184	0.384
18	0.006	0.16	0.39

Time (h)	T=4C	T=22C	T=35C
19	0.006	0.167	0.376

20	0.001	0.176	0.368
21	0	0.182	0.379
22	0	0.201	0.344
23	0.006	0.171	0.335
24	0.003	0.205	0.303

At times 0 and 24, dilutions of 1:1000, 10000, 100000, and 1000000 were made to determine the initial and final concentration of colony forming units. The dilutions were plated on agar plates and incubated overnight at 37℃. The resulting colonies were counted.

Temperature and Time	1:1,000 dilution	1:10,000 dilution	1:100,000 dilution	1:1,000,000 dilution
T=4C, T0	560	51	9	3
T=4C, T24	688	111	7	3
T=22C, T0	504	113	18	5
T=22C, T24	TNTC	2264	827	136
T=35C, T0	249	17	2	2
T=35C, T24	TNTC	TNTC	783	103

June 23

To try and boost the amount of linear DNA we had to work with, pcr of the rehydrated IDT BBI-GFP construct was carried out. This was done following IDT's protocol for PCR. Unfortunately, there were bands on the subsequent 1% agarose confirmation gel. We think the issue was that the annealing temperature was too high.

The pSB1C3-RFP plasmid that was transformed into TOP10 was minipred using the homebrew miniprep kit for the first time! And it worked! The yield was 36.6ng/uL with an A260/280 value of 1.90. The values were obtained from the nanodrop.

June 24

TOP that was transformed with BBI-GFP (June 21) was streaked onto Chlor plates and inoculated in Chlor broth. Both were left to grow overnight at 37℃. Another round of minipreps were done with the homebrew kit to extract pSB1C3-BBI #5-GFP. The end concentration was 63.6ng/uL with a 260/280 value of 1.93.

The PCR amplification of RFP was attempted but using the KAPA HiFi protocol. This makes more sense given that our reagents were from kapa and the protocol would be optimized for the reagents. The RFP was

used as a control to make sure that we had the right annealing temperature. The PCR was run using the following set up:

PCR steps:

2 min at 95C		1 cycle
30 s at 98 C	}	
30 s at 52C	}	30 cycles
1 min at 72C	}	
5 min at 72C		1 cycle

On a 1% agarose gel that was run to confirm the products, a band was seen at ~1600bp relative to the 1 kb plus ladder.

June 27

Using the transformed TOP10 we tried to confirm that BBI-GFP had been inserted into the bacteria, accomplished by colony PCR. Also amplified was the BBI-GFP now that the correct temperatures had been found. The same run settings were used as the previous day. There were eight samples amplified and then run on a 1% agarose gel for confirmation.

Lane 1	ladder
Lane 2	colony 1
Lane 3	colony 2
Lane 4	colony 3
Lane 5	colony 4
Lane 6	linear BBI #5-GFP
Lane 7	linear BBI #5-GFP
Lane 8	plasmidal BBI #5-GFP
Lane 9	plasmidal BBI #5-GFP

The strongest band observed was at 2100bp which was the size of our plasmid. The lanes with linear DNA only resulted in smears, which suggests either contamination or off target binding.

June 28

BBI #5-GFP in the pSB1C3 vector was sent down to the sequencing lab to be sequenced. Samples had to be prepared to the exacting standards of the sequencing lab. For each sample 3uL of template at 63.1ng/ul was used along with 1ul of Prefix-F or Suffix-R. The tubes were filled up to 12uL volume with ddH_2O.

More glycerol stocks of BBI #5-GFP were made.

June 28-July 1

Another round of growth curves was done over the course of four days. Bacteria was grown again at four different temperatures with growth being measured at hour time points.

Time (h)	T=4C	T=4C	T=22C	T=22C	T=35	T=35
0	0.003	0.005	0.004	0.003	0.01	0.024
1	0.002	0.003	0.008	0.008	0.014	0.029

2	0	0	0.009	0.005	0.034	0.069
3	0	0	0.011	0.006	0.06	0.136
4	0.0063	0.006	0.013	0.01	0.137	0.152
5	0.0055	0.0119	0.019	0.018	0.182	0.182
6	0.0059	0.0071	0.03	0.025	0.152	0.199
7	0.0064	0.0064	0.05	0.037	0.218	0.228
8	0.0058	0.007	0.0595	0.0575	0.2005	0.2655
9	0	0	0.076	0.098	0.222	0.246
10	0	0	0.103	0.113	0.264	0.271
11	0	0.0045	0.107	0.138	0.293	0.275
12	0.0039	0.0042	0.146	0.154	0.316	0.334
13	0.0038	0.0038	0.174	0.192	0.346	0.351
14	0.0043	0.004	0.208	0.206	0.367	0.368
15	0.0042	0.0046	0.193	0.227	0.397	0.56
16	0.0037	0.0041	0.2545	0.248	0.395	0.383
17	0	0	0.276	0.296	0.347	0.396
18	0	0	0.295	0.278	0.371	0.45

Time (h)	T=4C	T=4C	T=22C	T=22C	T=35C	T=35C
19	0.0049	0.0039	0.325	0.295	0.364	0..38
20	0.0032	0.0032	0.333	0.311	0.355	0.74
21	0.0033	0.0033	0.326	0.317	0.325	0.349
22	0.0041	0.0037	0.355	0.324	0.37	0.345
23	0.0038	0.0039	0.363	0.332	0.346	0.315
24	0.0034	0.0043	0.359	0.340	0.335	0.35

As per the first growth curve dilutions were made to determine the initial and final CFU concentration.

Temperature and Time	1:100	1:1,000	1:10,000	1:100,000	1:1,000,000
T=4C, T0	N/A	1180	177	7	1
T=4C, T0	N/A	1179	191	9	4
T=4C, T24	TNTC	711	63	N/A	N/A
T=4C, T24	TNTC	751	52	N/A	N/A
T=22C, T0	N/A	N/A	26	6	N/A
T=22C, T0	N/A	N/A	53	9	N/A
T=22C, T24	N/A	N/A	TNTC	267	52
T=22C, T24	N/A	N/A	TNTC	342	135
T=35C, T0	TNTC	904	75	N/A	N/A
T=35C, T0	TNTC	1960	172	N/A	N/A
T=35C, T24	N/A	N/A	1460	299	12
T=35C, T24	N/A	N/A	752	199	31

We tried to amplify BBI #5-GFP for the third time using the KAPA HiFi protocol. However VF2 and VR primers were used instead of the standard biobrick primers to see if the construct would be amplified from the pSB1C3.
The products were run on a 1% agarose gel for 45 minutes.

June 29
Stocks of ECE 103 and 153 arrived from Bacillus Genetic Stock Centre and overnight cultures of both were prepared in LB-Amp broth. Lots of media was made to account for the Amp and Kan markers used on these plasmids.

June 30
Glycerol stocks of the ECE 103 and 153 were made following standard protocol.
The integration vectors were mini preped from the two different *E. coli* strains.

E. coli strain	integration vector	concentration (ng/uL)	A260/280
ECE 103 (sample 1)	pMLK83	111.3	1.92

ECE 103 (sample 2)	pMLK83	146	1.84
ECE 153 (sample 1)	pSG1154	78.5	1.84
ECE 153 (sample 2)	pSG1154	188.4	1.89

Rai came by to help us amplify our constructs to try and fix what was going wrong. Both the RFP and BBI #5-GFP were amplified with a variety of different primers: BBK primers, biobrick standard, and verification primers. Following the amplification, the products were run on a 1% agarose gel. Also included was a confirmation digest of the pSB1C3 plasmid with RFP and BBI #5-GFP to ensure that it hadn't degraded. We followed Rai's procedure for that.

4 uL template

1 uL BSA

1 uL EcoRI

1 uL PstI

2 uL buffer (X10)

17 uL ddH2O

These samples were left to incubate at 37°C for 1 hour before heat shocking at 80°C for 20 min

Lane assignments for PCR/digestion products

Lane	GFP Gel	RFP Gel
1	BBI #5GFP using BBK primers	RFP using BBK primers
2	BBI #5GFP using BBK primers	RFP using BBK primers
3	BBI #5GFP using BBK primers	RFP using BBK primers
4	BBI #5GFP using BBK primers	RFP using BBK primers
5	Ladder (GeneRule 1Kb Plus)	Ladder (GeneRule 1Kb Plus)
6	BBI #5GFP using Prefix and Suffix primers	RFP using Prefix and Suffix primers
7	BBI #5GFP using Prefix and Suffix primers	RFP using Prefix and Suffix primers
8	BBI #5GFP using Prefix and Suffix primers	RFP using Prefix and Suffix primers
9	BBI #5GFP using Prefix and Suffix primers	RFP using Prefix and Suffix primers
10	Ladder (GeneRule 1Kb Plus)	Ladder (GeneRule 1Kb Plus)

11	BBI #5GFP using Verification primers	RFP using Verification primers
12	BBI #5GFP using Verification primers	RFP using Verification primers
13	BBI #5GFP using Verification primers	RFP using Verification primers
14	BBI #5GFP using Verification primers	RFP using Verification primers
15	pSB1c3-BBI #5GFP restriction digest	pSB1c3-RFP restriction digest

GFP Gel the results are as follows:

- BBK Primers: bands were seen at 1300 bp (expected = 1250 bp) and 300 bp
- Prefix and Suffix Primers: bands were seen at 1100 bp (expected = 970 bp)
- VerificatioPrimers: faint bands were seen at 1300 bp (expected = 1221 bp) and 400 bp
- Restriction digest: one faint band was seen at 2100bp (not enough DNA was present)

RFP results

- There were no bands

We got a new strain of *E. coli* from Deirdre: DB3.1. We wanted to use this strain as it is resistant to the *ccdB* gene which is a component of the new pSB3C5 (low copy number) plasmid we want to use. Low copy number hopefully will mimic having the BBI integrated into the bacillus genome at several different positions. We don't have the time or the expertise to integrate multiple different constructs into the *Bacillus* genome. The bacteria were streaked onto an LB plate and incubated overnight at 37℃.

July 4

Overnight cultures of *E. coli* DB3.1, ECE103. ECE153, RFP and BBI #5GFP were prepared in 7mL of LB, LB-Amp, -Chlor respectively. The overnights were incubated at 37℃ with shaking at 200rpm.

Master plates of *E. coli* DB3.1, ECE103 and ECE153 were plated stored at 4℃.

Dilutions of 1:1, 1:10, 1:100, 1:1,000, 1:10,000, 1:100,000 and 1:1,000,000 of frozen *B. subtilis* samples s3 taken at time T8 at temperatures of 4℃, 22℃ and 35℃ were spot-plated (20uL) on LB plates and left to incubate O/N at 37℃ to determine the presence of spores in each sample.

We digested pSB1c3-RFP, pSB1c3-BBI #5GFP, pMLK83 and pSG1154 using Rai's protocol.

	BBI #5GFP	RFP	pMLK83	pSG1154
single digest	PstI	PstI	HinDIII	HinDIII
"double" digest	PstI + EcoRI	PstI + EcoRI	EcoRI	XbaI

The digested products were run on a 1% agarose gel and run at 120 V for 45 min:

Lane	Contents	Expected bands (Kb)	Observed bands

1	1 Kb Plus GeneRuler ladder		none
2	pSB1c3-BBI #5GFP/PstI	3.1	none
3	pSB1c3-RFP/PstI	3.2	none
4	pMLK83/HinDIII	9.9	none
5	pSG1154/HinDIII	7.6	none
6	pSB1c3-BBI #5GFP/PstI+EcoRI	1, 2.1	none
7	pSB1c3-RFP/PstI+EcoRI	1.1, 2.1	none
8	pMLK83/EcoRI	2, 7.9	none
9	pSG1154/XbaI	1, 1.6, 5	none

July 5
The frozen spores from yesterday grew and 1:10000 dilution is the most appropriate for spot plating.

The DB3.1 cells that we obtained from Deirdre were made competent following the chemical competency protocol for *E. coli*.

Using the overnight cultures: pSB1c3-BBI #5GFP, pSB1c3-RFP, pMLK83 and pSG1154 were double digested with EcoRI and XbaI while pSB1C3 with RFP or BBI #5-GFP were singly digested with EcoRI as well.

July 6
pSB1c3-BBI #5GFP, pSB1c3-RFP, pMLK83 and pSG1154 were prepped from *E. coli* using the Omega and homebrew kits (protocol on page 3 of Rachelle's lab book) for comparison. The plasmid yield was determined with the nanodrop spectrophotometer.

Plasmid	Omega concentration (ng/uL)	Omega A260/280	Homebrew concentration (ng/uL)	Homebrew A260/280
pSB1c3-BBI #5GFP	45.7	1.89	171.1	1.86
pSB1c3-RFP	94.6	1.86	120.4	1.85

pMLK83	38.9	1.86	99	1.85
pSG1154	20.9	2.16	137.1	1.87

The plasmids were the sent for sequencing. We sent down 6 samples of pSB1c3-BBI #5GFP; 3 samples contained the forward Prefix-F primer and 3 samples contained the reverse Suffix-R primer. However, we hit a snag :(We need to reapply for our sequencing authorization, so we're waiting for Dr. Mayi to come in from her vacation to sign forms.

The restriction digests from yesterday (July 5) and the minipreps from today were run on a 1% agarose gel at 120 V for 45 min:

Lane	Contents of well	Expected band size (kb)	Observed band size (kb)
1	GeneRuler 1KB Plus ladder		
2	pMLK83 from Omega kit digest: EcoRI+XbaI	2, 7.9	2, 7.5
3	pSG1154 from Omega kit digest: EcoRI+XbaI	1, 1.6, 5	1.5, 5
4	pSB1c3-RFP from Omega kit digest: EcoRI + XbaI	3.1	3.1
5	pSB1c3-BBI #5GFP from Omega kit digest: EcoRI + XbaI	3.1	3.1
6	pSB1c3-BBI #5GFP from homebrew digest: EcoRI	3.1	3.1
7	pSB1c3-BBI #5GFP digest: EcoRI	3.1	3.1
8	pSB1c3-RFP from homebrew digest: EcoRI	3.1	>20
9	pSB1c3-RFP from homebrew digest: EcoRI	3.1	>20
10	pSB1c3-BBI #5GFP undigested from homebrew	?	2.5
11	pSB1c3-RFP undigested from homebrew	?	6
12	pMLK83 undigested from homebrew	?	10
13	pSG1154 undigested from homebrew	?	7
14	GeneRuler 1KB Plus ladder		

Almost everything worked! We reprepped the RFP plasmid...

We transformed the DB3.1 cells with the pSB3C5 plasmid that was in well 4D of kit plate four in the 2014 distribution kit.
Two different types of media were made in preparation for the next set of growth curves

2X LB Media - in 1 L dH2O

2% tryptone (20 g)

1% yeast extract (10 g)

2% NaCl (20 g)

Super-Rich Media - in 1 L dH2O

2.5% tryptone (25 g)

2% yeast extract (20 g)

0.3% K2HPO4 (3 g)

3% lucose (150 mL of 20%)

Mix all components except glucose and adjust pH to 7.5

Autoclave solution except glucose

Filter sterilize glucose and add to cooled solution

If pH<7, add 1.5 mL of 6M NH4OH per litre to raise pH

July 7

A restriction digest of pMLK83 and pSG1154 was performed in separate reaction tubes. pMLK83 was digested using EcoRI and XbaI and pSG1154 was digested using EcoRI and SpeI following Rai's protocol (on page 31 of Rachelle's lab book); **we realized too late that the digest of pMLK83 wouldn't allow for our insert to be ligated because both EcoRI and XbaI are in the prefix**...

Another confirmation digest was run for the four plasmids we were working with: -pMLK83, pSG1154, pSB1c3-RFP and pSB1c3-BBI #5GFP. The products were then run on a 1% gel

Lane	Contents of the well	Expected band size (kb)	Observed band size (kb)	Explanation
1	1KB Plus GeneRuler ladder			
2	pMLK83 digested with PstI	4.4, 5.5	4.4, 5.5	
3	pMLK83 digested with PstI	4.4, 5.5	4.4, 5.5	
4	pSG1154 digested with PstI	3.4, 4.2	4.2, 7.6	nicked plasmid => 7.6 kb, 3.4 was too faint to visualize

5	pSG1154 digested with PstI	3.4, 4.2	4.2, 7.6	nicked plasmid => 7.6 kb, 3.4 was too faint to visualize
6	pSB1c3-RFP digested with PstI+EcoRI	1.1, 2.1	3.1	no EcoRI was added
7	pSB1c3-RFP digested with PstI+EcoRI	1.1, 2.1	1.1, 2.1	
8	pSB1c3-BBI #5GFP digested with PstI+EcoRI	1, 2.1	3.1	no EcoRI was added
9	pSB1c3-BBI #5GFP digested with PstI+EcoRI	1, 2.1	1, 2.1	
10	1KB Plus GeneRuler ladder			

All 4 plasmids were confirmed! Yay!

July 8
We finally made transformation media. We first made stock solutions of everything and autoclaved them except for the glucose, which was filter sterilized. We followed the recipe provided to us by Dr. Wong.

We realized that digesting the *ComK* insert with EcoRI and XbaI wouldn't work as both of the restriction sites are in the prefix region. The digestion was redone, following Rai's protocol. The products were then ligated into the pMLK83 vector following the usual protocol.

July 9
The media test growth curves have started! A 7mL of LB-hygro was inoculated with *B. subtilis* at 4:45pm and incubated at 37℃ and 200rpm overnight.

2 aliquots of TOP10 were transformed he newly-ligated pMLK83-*ComK* and pSG1154-*ComK* plasmids following the standard procedure. 10ul of each DNA sample was used and the transformed cells were plated on Amp plates. The bacteria were then grown up overnight at 37℃

July 11
We started measuring the growth of *B. subtilis* but every 12 hours we added a 1mL of media. The addition of media was to simulated popping one of the additional media packets in the patch. Each round of growth curves a different type of media was added. The experiments were set up the same as the previous growth curves. A 50mL falcon tube with 9mL of hygromycin media was incubated with 1 mL of culture and grown at 35℃ and 200rpm. The four different treatments were: nothing added, plain LB, 2x concentration of LB, and

super rich media. As before, at the start and end of the experiments, dilutions of the culture are made to determine initial and final CFU (colony forming units).

Adding super rich media every twelve hours:

Time (h)	0-8 h (1)	0-8 h (2)	8-16 h (1)	8-16 h (2)	16-24 h (1)	16-24 h (2)
T0	0.026	0.020	0.146	0.132	0.208	0.233
T1	0.031	0.028	0.170	0.161	0.244	0.313
T2	0.053	0.041	0.183	0.177	0.282	0.389
T3	0.099	0.098	0.288	0.215	0.263	0.312
T4	0.113	0.153	0.239	0.249	0.240	0.336
T5	0.142	0.126	0.255	0.245	0.324	0.342
T6	0.181	0.175	0.243	0.289	0.337	0.380
T7	0.192	0.187	0.293	0.297	0.338	0.339
T8	0.226	0.221	0.355	0.309	0.339	0.363

All OD measurments were made at 1:10 dilution unless otherwise stated.

July 12

A 1% agarose gel was run to confirm that the extracted plasmids were not degrading while in storage. The four plasmids digested were: pMLK83, pSG1154, pSB1C3+BBIGFP, and pSB1C3+RFP. All plasmids were singly and doubly digested. The ladder used was the GenRuler 1KB plus and the gel was run for 45 minutes at 120V. The gel ran quite weirdly, bands seemed to be off and the ladder looked like it had been degraded. The gel was re-done just in case.

We tried to PCR out Comk from pMLK83 and pSG1154 transformed into TOP10. A 25ul reaction protocol was used instead of 50ul. 8 colonies from each plate were selected for 16 total reactions and 17x master mix. The KAPA PCR protocol was used but adjusted to the smaller volume.On the confirmation gel, there were no bands for either of the pMLK83 or SG1154. The ladder used was the GenRuler 1kb plus which was very faint.

Growth curves with super rich media continues:

Time (h)	Tube 1 24-32 h (1)	Tube 2 24-32 h (2)	Tube 3 32-40 h (1)	Tube 4 32-40 h (2)	Tube 5 40-48 h (1)	Tube 6 40-48 h (2)
T9	0.071	0.056	0.063	0.060	0.058	0.054
T10	0.041	0.048	0.039	0.040	0.037	0.048

T11	0.045	0.050	0.047	0.066	0.044	0.048
T12	0.047	0.061	0.044	0.047	0.050	0.059
T13	0.086	0.065	0.050	0.049	0.049	0.058
T14	0.054	0.063	0.041	0.069	0.058	0.058
T15	0.059	0.69	0.057	0.047	0.041	0.055
T16	0.053	0.072	0.049	0.066	0.067	0.068
T17	0.055	0.058	0.045	0.050	0.052	0.050

*all measurements are made in a 1:100 dilution unless stated otherwise

Initial Cell Count - CFU per 100 uL

CFU (in 100 uL)	**1:1,000 dilution**	**1:10,000 dilution**
Tube 1, T0	TNTC	522
Tube 2, T0	TNTC	424

July 13

More colony PCR of the TOP10 transformed with pMLK83+*ComK* and pSG1154+*ComK*. Colonies 9-24 were selected on both plates for a total of 32 reactions and master mix of 33x. Same protocol as yesterday. The products were run on gel and the bright bands ⅔ of the way down the gel are approximately 700-800bp (above the empty space where the loading dye is). There is another series of bands at the 1000-1200 bp range. The second series of bands is the range of interest as *ComK* is ~1200 base pairs. The large amount of smaller bands is worrying as it indicates that the primers could be annealing to off-target sites. We aren't sure as the plasmid sequences are not available.

The pSB3C5, which contained BBa_I52001 (*Cccd*), was minipreped from DB3.1 following the homebrew protocol. The yield was 545.7ng/ul with a A260/280 of 1.82.

All the plasmids were digested again because we are paranoid that the plasmids might be being degraded by a nucleases in the water we were using for resuspending the pellet during minipreps. We followed Rai's protocol and ran the products on an 0.8% agarose gel at 100v for 45 minutes. All of our plasmids are now confirmed.

Lane	Well contents	Expected bands (kb)	Observed bands (kb)
1	1 Kb Plus GeneRuler DNA ladder		
2	pMLK83 digested with XbaI	9.9	9,9

3	pSG1154 digested with EcoRI	7.6	7.6
4	pSB1c3-RFP digested with EcoRI	3.1	3.1
5	pSB1c3-BBI #5GFP digested with EcoRI	3.1	3.1
6	pSB3c5-I52001 digested with EcoRI	3.8	3.8
7	pMLK83 digested with EcoRI + PstI	2, 3.5, 4.4	2, 3.5, 4.4
8	pSG1154 digested with EcoRI + PstI	3.4, 4.2	**2.5, 5**
9	pSB1c3-RFP digested with EcoRI + PstI	1.1, 2.1	1.1, 2.1
10	pSB1c3-BBI #5GFP digested with EcoRI + PstI	1, 2.1	1, 2.1
11	pSB3c5-I52001 digested with EcoRI + PstI	1.1, 2.7	1.1, 2.7
12	1 Kb Plus GeneRuler DNA ladder		

Continuation of *B. subtilis* growth curve with super rich media:

Time (h)	Tube 1 48-56 h (1)	Tube 2 48-56 h (2)	Tube 3 56-64 h (1)	Tube 4 56-64 h (2)	Tube 5 64-72 h (1)	Tube 6 64-72 h (2)
T18	0.051	0.074	0.046	0.051	0.060	0.043
T19	0.056	0.076	0.047	0.050	0.058	0.042
T20	0.056	0.077	0.052	0.062	0.057	0.050
T21	0.071	0.085	0.058	0.060	0.060	0.043
T22	0.064	0.072	0.048	0.069	0.051	0.042
T23	0.060	0.074	0.046	0.088	0.097	0.038
T24	0.116	0.135	0.096	0.107	0.107	0.088
T25	0.120	0.142	0.095	0.095	0.100	0.085
T26	0.134	0.143	0.096	0.104	0.106	0.095

*all measurements are made in a 1:100 dilution unless stated otherwise

July 14

Due to issues with the integration vectors, pMLK83 and pSG1154, we tried to to ligate the linear *ComK* into pSB1C3 as a backup plan. The *ComK* construct contains integration sites flanked by the prefix and suffix sequences allowing it to still be able to integrate into the *Bacillus* genome.

The digestions were done following Rai's protocol with only 1ul of *ComK* to save materials and 12ng/ul of pSB1C3. The insert and vector were ligated together using the usual protocol. Nick and Miriam did the digest and restriction so that they knew what to do when James and Rachelle are away.

The ligated products were subsequently transformed into TOP10 following the standard transformation procedure. However the one hour recovery period occurred at 35°C instead of 37°C as the shaker had not warmed up enough. The 50uL of the transformants were plated onto LB-Chlor plates.
The last day of the super rich growth curve! First day of using 2xLB!

Time	2X LB 0-8 h (1)	2XLB 0-8 h (2)	2XLB 0-8 h (3)	SR 0-8 h (3)	2XLB 8-16 h (1)	2XLB 8-16 h (2)	2XLB 8-16 h (3)	SR 8-16 h (3)	2XLB 16-24 h (1)	2XLB 16-24 h (2)	2XLB 16-24 h (3)	SR 16-24 h (3)
T0	0.006	0.005	0.007	0.005	0.129	0.066	0.134	0.126	0.219	0.185	0.233	0.157
T1	0.014	0.009	0.007	0.004	0.138	0.093	0.151	0.151	0.244	0.193	0.247	0.228
T2	0.042	0.021	0.023	0.020	0.156	0.109	0.156	0.161	0.241	0.234	0.275	0.254
T3	0.077	0.058	0.069	0.055	0.158	0.122	0.163	0.163	0.269	0.235	0.276	0.259
T4	0.119	0.101	0.089	0.095	0.181	0.140	0.186	0.194	0.287	0.244	0.299	0.275
T5	0.117	0.112	0.114	0.101	0.179	0.154	0.179	0.192	0.287	0.240	0.291	0.294
T6	0.130	0.128	0.125	0.123	0.204	0.181	0.210	0.235	0.310	0.266	0.3915	0.317
T7	0.139	0.147	0.154	0.139	0.230	0.266	0.230	0.284	0.318	0.291	0.312	0.318
T8	0.161	0.167	0.167	0.234	0.245	0.228	0.247	0.343	0.347	0.304	0.367	0.330

*all measurements are made in a 1:10 dilution unless stated otherwise
Initial cell counts:

CFU (100 uL)	**1:1,000 dilution**	**1:10,000 dilution**
2XLB, (1), T0	TNTC	79
2XLB (2), T0	contaminated	43

2XLB (3), T0	397	50
SR (3), T0	TNTC	39

July 15

The transformations from yesterday didn't work, there was no growth on any of the plates. We replated the transformants but used 100uL instead of 50uL. The cells were incubated at 37℃ overnight.

The last 11 colonies from the both the transformed MLK83 and SG1154 plates were PCRed to see if *ComK* was inserted into either of the integration vectors. The same procedure from July 12 was followed except that a 24x master mix was used instead of the 33x. The products were run on a 1% agarose gel for 25 minutes at 120v to confirm the PCR.

According to the bands on the gel, the primers amplified a small segment of dna less than 500bp in size. The second most prominent band is again around the 700 bp marker of the ladder. Where we expect to see *Comk*, 1200bp, is a very weak band. Again there appear to be primer issues lots of small products were generated including in the water control which should not have any DNA. The PCR products were run a second time on different gels to see if running a gel with only one well set would change the results. It didn't.

Continuation of Super Rich and 2xLB

Time	2xLB 1	2xLB 2	2xLB 3	SR 4	2xLB 5	2xLB 6	2xLB 7	SR 8	2xLB 9	2xLB 10	2xLB 11	SR 12
T9	0.262	0.316	0.295	0.300	0.307	0.282	0.303	0.073 *	0.339	0.321	0.332	0.389
T10	0.234	0.271	0.251	0.280	0.326	0.273	0.302	0.0495*	0.307	0.320	0.31	0.365
T11	0.239	0.275	0.245	0.293	0.314	0.276	0.297	0.053 *	0.325	0.340	0.329	0.376
T12	0.275	0.286	0.243	0.340	0.331	0.288	0.308	0.070 *	0.350	0.342	0.324	0.041 *
T13	0.276	0.323	0.302	0.295	0.339	0.281	0.306	0.086 *	0.052 *	0.341	0.333	0.027 *
T14	0.262	0.352	0.304	0.377	0.330	0.276	0.282	0.063 *	0.053 *	0.333	0.302	0.040 *
T15	0.302	0.342	0.328	0.371	0.295	0.255	0.274	0.070 *	0.029 *	0.303	0.315	0.039 *
T16	0.319	0.386	0.387	0.045 *	0.307	0.304	0.323	0.055 *	0.037 *	0.331	0.339	0.052 *
T17	0.323	0.354	0.325	0.049	0.314	0.294	0.245	0.086	0.035	0.039	0.289	0.066

				*				*	*	*		*

All measurements were at 1:10 dilutions apart from the *measurements which were at 1:100.

The DNA sequences for the indicator systems were rehydrated from a number of the kit plates. The parts rehydrated were BBa_K091110 and BBa_K909006 from the 2012 and 2016 kit plates respectively. The two parts were transformed into TOP10 with two aliquots each.

July 16

Time	2xLB 1	2xLB 2	2xLB 3	SR 4	2xLB 5	2xLB 6	2xLB 7	SR 8	2xLB 9	2xLB 10	2xLB 11	SR 12
T18	0.292	0.303	0.284	0.039 *	0.261	0.356	0.300	0.074 *	0.227	0.036 *	0.189	0.031 *
T19	0.268	0.301	0.283	0.053 *	0.265	0.352	0.283	0.067 *	0.225	0.056 *	0.192	0.049 *
T20	0.274	0.296	0.312	0.051 *	0.266	0.360	0.300	0.066 *	0.222	0.098 *	0.201	0.029 *
T21	0.268	0.291	0.279	0.057 *	0.263	0.363	0.288	0.067 *	0.209	0.075 *	0.183	0.036 *
T22	0.257	0.287	0.268	0.078 *	0.266	0.351	0.300	0.072 *	0.213	0.067 *	0.216	0.008 *
T23	0.258	0.283	0.289	0.083 *	0.311	0.372	0.304	0.048 *	0.276	0.135 *	0.204	0.025 *
T24	0.262	0.300	0.300	0.069 *	0.284	0.375	0.314	0.066 *	0.230	0.021 *	0.208	0.019 *
T25	0.248	0.283	0.268	0.050 *	0.254	0.363	0.302	0.066 *	0.221	0.074 *	0.189	0.059
T26	0.260	0.304	0.273	0.065 *	0.256	0.333	0.263	0.100 *	0.240	0.063 *	0.226	0.045 *

All measurements were at 1:10 dilutions apart from the *measurements which were at 1:100.

Bacillus subtilis was transformed with pSB1C3-RFP and pSB1C3-BBI #5GFP. The transformation protocol was provided by Dr. Wong. Between 120ng and 200ng of plasmid was used for the various transformations. 250uL of transformed cells were plated onto LB-Chlor plates and incubated overnight at 37℃.

July 18

No addition of media

Time	1	2	3	4	5	6	7	8	9
T0	0.002	0.002	0.004	0.139	0.142	0.153	0.226	0.232	0.245
T1	0.024	0.016	0.009	0.168	0.164	0.165	0.245	0.247	0.267
T2	0.015	0.002	0.001	0.166	0.184	0.192	0.278	0.285	0.302
T3	0.010	0.012	0.003	0.079	0.196	0.232	0.273	0.286	0.298
T4	0.012	0.013	0.001	0.187	0.221	0.241	0.308	0.323	0.326
T5	0.018	0.011	0.010	0.282	0.274	0.269	0.365	0.341	0.356
T6	0.011	0.005	0.011	0.244	0.309	0.313	0.383	0.373	0.374
T7	0.019	0.014	0.022	0.271	0.317	0.322	0.363	0.353	0.350
T8	0.013	0.013	0.041	0.241	0.328	0.354	0.362	0.364	0.383

All measurements were made at a 1:10 dilution

CFU (100ul)	1:1000	1:10 000
T0	70	41
T0	158	TNTC
T0	169	548

The *Bacillus* transformations were successful, there were lots of colonies growing on the LB-Chlor plates.

July 19

Time	1	2	3	4	5	6	7	8	9
T9	0.234	0.088	0.221	0.256	0.237	0.229	0.184	0.188	0.199
T10	0.199	0.180	0.255	0.245	0.238	0.224	0.202	0.200	0.195
T12	0.230	0.157	0.260	0.274	0.238	0.221	0.199	0.221	0.197
T13	0.265	0.186	0.283	0.223	0.211	0.196	0.199	0.224	0.179
T14	0.296	0.222	0.292	0.216	0.204	0.102	0.193	0.191	0.103
T15	0.313	0.226	0.289	0.218	0.215	0.189	0.169	0.190	0.170
T16	0.340	0.236	0.310	0.209	0.232	0.172	0.171	0.162	0.157
T17	0.353	0.256	0.203	0.171	0.179	0.170	0.156	0.151	0.157

All measurements were at a 1:10 dilution

pSB1C3-RFP and pSB1C3-BBIGFP were minipreped from the transformed *B. subtilis* and the pMLK83-*Comk* and pSG1154-*ComK* were minipreped from TOP10 using the homebrew miniprep kit.

Plasmid	Yield (ng/ul)	A260/280
pSB1C3-BBIGFP	50	1.50
pSB1C3-BBIGFP	96.1	1.56
pSB1C3-RFP	49.2	1.51
pSB1C3-RFP	40.7	1.57
pMLK83-*Comk*	59.9	1.74
pMLK83-*Comk*	240.2	1.90
pSG1154-*ComK*	334.2	1.89
pSG1154-*ComK*	373	1.84

The Plasmids were then digested to confirm their identity

Plasmid	Single digest	Double digest
pSB1C3-BBIGFP	EcoRi	EcoRi+PstI
pSB1C3-RFP	EcoRi	EcoRi+PstI
pMLK83-*ComK*	XbaI	EcoRi+PstI
pSG1154-*ComK*	EcoRi	EcoRi+PstI

Rai's protocol was used

July 20

Time	1	2	3	4	5	6	7	8	9
T15	0.278	0.294	0.247	0.168	0.160	0.158	0.131	0.131	0.159
T19	0.270	0.288	0.253	0.173	0.170	0.172	0.138	0.149	0.175
T20	0.250	0.270	0.244	0.145	0.153	0.132	0.135	0.146	0.152
T21	0.234	0.249	0.224	0.171	0.171	0.131	0.119	0.137	0.151
T22	0.249	0.260	0.234	0.165	0.152	0.152	0.121	0.120	0.148

T23	0.215	0.228	0.228	0.167	0.157	0.153	0.112	0.133	0.150
T24	0.220	0.228	0.221	0.164	0.154	0.148	0.132	0.126	0.159
T25	0.207	0.225	0.216	0.163	0.161	0.169	0.118	0.125	0.152
T26	0.206	0.218	0.210	0.160	0.151	0.144	0.116	0.120	0.147

All measurements were at a dilution of 1:10

The products of the restriction digests from yesterday were run on a 0.8% gel at 100v for 40 minutes. The vast majority of lanes had no bands and the lanes that did have bands were between 4 and greater than 10kb in size. Clearly these did not work.

July 21

Time	1	2	3	4	5	6	7	8	9
T0	0.004	0.001	0.002	0.003	0.003	0.089	0.191	0.131	0.169
T1	0.010	0.004	0.009	0.005	0.004	0.118	0.225	0.170	0.198
T2	0.011	0.003	0.004	0.001	0.001	0.135	0.236	0.182	0.212
T3	0.028	0.022	0.014	0.004	0.003	0.136	0.249	0.198	0.237
T4	0.061	0.046	0.045	0.001	0.010	0.165	0.263	0.208	0.236
T5	0.101	0.103	0.105	0.003	0.000	0.160	0.278	0.241	0.269
T6	0.133	0.117	0.117	0.006	0.003	0.189	0.292	0.212	0.265
T7	0.134	0.127	0.134	0.005	0.004	0.165	0.312	0.229	0.257
T8	0.170	0.156	0.155	0.011	0.004	0.182	0.295	0.242	0.262

All measurements were made at 1:10 dilution

July 22

Time	1	2	3	4	5	6	7	8	9
T9	0.171	0.149	0.153	0.162	0.119	0.243	0.203	0.202	0.199
T10	0.210	0.198	0.193	0.195	0.154	0.248	0.209	0.208	0.200
T11	0.234	0.209	0.211	0.228	0.194	0.258	0.199	0.204	0.188
T12	0.239	0.26	0.223	0.258	0.211	0.234	0.203	0.202	0.191
T13	0.300	0.246	0.245	0.278	0.59	0.251	0.191	0.186	0.175

T14	0.352	0.280	0.283	0.317	0.273	0.266	0.197	0.186	0.180
T15	0.332	0.264	0.278	0.291	0.223	0.209	0.162	0.161	0.158
T16	0.320	0.292	0.286	0.312	0.253	0.204	0.171	0.149	0.151
T17	0.311	0.272	0.271	0.313	0.276	0.186	0.157	0.150	0.144

July 23

Finished growth curves!

	1(0:00)	2 (:05)	3 (:10)	4 (:15)	5 (:20)	6 (:25)	7 (:30)	8 (:35)	9 (:40)
T0	0.236	0.170	0.146	0.191	0.201	0.084	0.084	0.089	0.077
T1	0.248	0.161	0.150	0.185	0.208	0.089	0.085	0.088	0.072
T2	0.241	0.157	0.137	0.150	0.209	0.093	0.089	0.088	0.076
T3	0.233	0.154	0.141	0.168	0.220	0.087	0.089	0.094	0.081
T4	0.222	0.151	0.136	0.172	0.244	0.088	0.093	0.091	0.085
T5	0.214	0.155	0.146	0.172	0.234	0.088	0.100	0.096	0.081
T6	0.215	0.152	0.143	0.162	0.201	0.077	0.086	0.088	0.073
T7	0.226	0.141	0.129	0.155	0.221	0.084	0.091	0.084	0.081
T8	0.206	0.137	0.123	0.158	0.201	0.077	0.099	0.091	0.076

July 25

Comk was digested and ligated into pSB1C3. The ligations were stored overnight at 4℃ in preparation for transformations the next day.

The BBI constructs for *Bacillus* were rehydrated following the IDT protocol. The final concentration was between 10-50uL. The constructs were then digested and ligated into pSB1C3 and then transformed into TOP10 which was then incubated overnight at 37℃.

July 26

The transformations with the *ComK* containing plasmids were inconclusive and we decided to redo everything the next day.

The BBI #1 with restriction enzyme sites (RE) and BBI #1 without RE transformations were successful, isolated purple colonies were seen for both. BBI #5 with RE and BBI #5 without RE transformations weren't successful, no colonies grew on the plates.

> Note: We revisited these results later in august and september and realised that these original transformations were actually not successful. The purple colour was due to RFP that was not cut out of the pSB1C3

Digestion, ligation, and transformation was repeated for the BBI #5 constructs. Lastly master plates for cPCR were made for both of the BBI #1 constructs.

July 27
Transformations of TOP10 with *ComK* inserted into the integration vectors was successful. The transformation with pSG1154+*ComK* may have not been successful given that there was only one colony on the plate. IT was discovered afterwards that pMLK83 and pSG1154 plasmids had Amp resistance and Chlor.

The *ComK* with *AmyE* integration sequences was rehydrated following the instructions from IDT. The rehydrated construct was then digested following Rai's protocol. 2-4uL of vector (pSG1154 and pMLK83 respectively) was used but only 2uL of insert was used to save on the limited amount of DNA we had to work with. Lastly, the construct in the TOP10 and plated on Amp. plates. A negative control of untransformed TOP10 was plated as well.

The BBI #5 transformations attempt #2 wasn't successful again. The plates were left in the incubator, in the hope that colonies would grow with more time. Colonies were observed after a while.

Finally, overnight cultures of BBI #1 with RE were made and incubated at 37℃.

July 28
We discovered that the concentration of Amp. was not strong enough as the negative control, untransformed TOP10 was able to grow after being plated yesterday. Thus, new Amp. plates were made with the new Amp. was delivered.

The overnight cultures from July 27 were minipreped using the homebrew kit. Five cultures were used:

Colony #	Plasmid	Yield(ng/ul)	A260/280
1	pSB1C3-BBI #1 RE	8.6	1.33
7	pSB1C3-BBI #1 RE	6.1	1.34
8	pSB1C3-BBI #1 RE	15.4	1.48
10	pSB1C3-BBI #1 RE	14.2	1.48
11	pSB1C3-BBI #1 RE	16.1	1.59

The other colonies (12-22) of the master plates for the TOP10 transformed with BBI #1 constructs were cPCRed and the products were run on the a 1% gel.
Master plates for the now grown BBI #5 constructs were made and incubated at 37℃ overnight.

July 29

Both of the BBI #5 constructs were cPCRed off the master plates from yesterday with the products run a gel for confirmation. Unfortunately there were no bands on the gel, maybe a problem with the PCR.
The same minipreps from yesterday were redone, again using the homebrew kit. Also included were two colonies from BBI #1 w/o RE

Colony #	Plasmid	Yield	A260/280
1	BBI 1 w/t RE	10.1	2.71
7	BBI 1 w/t RE	16.7	2.32
8	BBI 1 w/t RE	45.1	1.99
10	BBI 1 w/t RE	68.8	2.02
11	BBI 1 w/t RE	44.3	2.05
18	BBI 1 w/o RE	78.3	1.98
20	BBI 1 w/o RE	80.7	1.97

August 2
The TOP10 transformed with either pSG1154-*ComK* or pMLK83-*ComK* grew on the Amp. plates. These *ComK* constructs contained the AmyE integration sequences. Master plates of these colonies were made so that PCR could be performed tomorrow.

The BBI #5 with and without RE were cPCRed. The colonies selected were: BBI #5 w/t RE 1-5 and BBI #5 w/o RE 1&2. On the confirmation gel, there were no bands. More overnights of both BBI 5 with and without restriction enzymes were made.

August 3
The Homebrew of BBI #1 were redone for the third time...Also included were the BBI #5 constructs.

Colony	Plasmid	Yield (ng/uL)	A260/280
1	BBI #1 with RE	5	1.42
7	BBI #1 with RE	4.3	1.04
8	BBI #1 with RE	8.4	1.47
10	BBI #1 with RE	4.7	1.37
11	BBI #1 with RE	25.9	1.78
18	BBI #1 without RE	26.3	1.56
20	BBI #1 without RE	40.0	1.62

5	BBI #5 with RE	25.8	1.71
1	BBI #5 without RE	17.8	1.74

TOP10 was transformed with the two different chromoproteins used for our indicator system. The constructs are blue and pink chromoproteins with one of three promoters: BBa_K78003, Pveg, PsspB; BBa_K780003, Pveg, PsspB respectively. In total there were 6 different constructs.
Lastly, *B. subtilis* was streaked onto a LB plate and incubated overnight at 37 °C.

August 4
B. subtilis was transformed with pSB3C5 plasmids containing BBI #1 or #5 with and without Restriction sites. *B. subtilis* was transformed following Dr. Wong's Protocol. Only one tube of SP1 media was inoculated with *B. subtilis* overnight culture as there were minimal number of colonies on the streak plate. The one tube of SP1 culture was split into 4 tubes each containing 4.5mL of SP2 media and 0.5mL of SP1 culture. 1 mL of SP2 culture from each tube was used for 2 transformations total. 20uL of DNA was used to transform the cells, with amount of DNA between 0.3-1 ug. 4 different constructs were used for two repeats each.

cPCR using TOP10 cells transformed with pMKL83-*ComK* was done. The cells were taken from the master plates made on August 2. Unfortunately only colony #15 was able to grow. 8 reactions total were run with a 9x master mix. Protocol used was the same from July 12.

The August 3 minipreps were redone as it was thought that the yields were too but this attempt didn't go that well either…

Colony	Plasmid	Yield (ng/ul)	A260/280
1	BBI #1 with RE	18.9	1.45
7	BBI #1 with RE	13.9	1.49
8	BBI #1 with RE	16.4	1.97
10	BBI #1 with RE	24.8	1.24
11	BBI #1 with RE	24.4	1.28
18	BBI #1 without RE	26.1	1.31
20	BBI #1 without RE	26.1	1.24
5	BBI #5 with RE	26.7	1.32
1	BBI #5 without RE	2.1	0.77

The extracted plasmids were then digested and run on a gel for confirmation. No DNA was seen in the gel unfortunately.
The TOP10 that were transformed with the chromoproteins did not grow and were then left to keep growing in the incubator at 37°C.

August 5
Samples of all four BBI constructs was sent to the sequencing for a more precise conformation relative to running PCR or restriction digest products on a gel.

August 8
The *Bacillus* cells that were transformed on August 4 were cPCRed. Eight samples were taken from each plate except for two plates that needed more time to grow (these were *Bacillus* transformed with BBI 5 constructs). From the plates that took longer to grow, 4 and 2 samples were used. In total a 57x master mix was needed for the 56 samples run. The same protocol from July 12 was used.

August 9
Overnight cultures of the transformed *Bacillus* from August 4 were minipreped using the Omega-BioTek E.Z.N.A plasmid kit. The kit protocol was followed except that 10mL of culture was used for each of the 4 plasmids. This was to compensate the generally lower yields that we were getting from *Bacillus.* The Omega-BioTek E.Z.N.A plasmid kit was used as we wanted to send these plasmids down for sequencing and wanted the higher quality preps.

Plasmid	Yield (ng/ul)	A260/280
pSB3C5 BB1 #5 w/o RE	33.2	1.86
pSB3C5 BB1 #5 w/t RE	30.3	1.99
pSB3C5 BB1 #1	27.2	1.93
pSB3C5 BB1 #1	44.5	1.94

The six different chromoprotein constructs (blue or pink chromoproteins with either Pveg, PsspB, or BBa_K780003 promoters) were double digested with EcoRI and PstI. The constructs were then inserted into both pSB1C3 and pSB3C5 (both digested with EcoRI and PstI). The products were ligated and transformed into TOP10. The transformed cells were incubated at 37℃ overnight.

August 10
Prepared eight samples for sequencing. There were two samples for each of the plasmids, forward and reverse. 150ng of template were used for each of the samples in order to be within the 50-100ng range/1 kb. 1uL of primer was used for each sample, either prefix-forward or suffix reverse depending on whether the sample was forward or reverse. The remaining volume was filled to 12uL with water.

The transformed TOP10 from yesterday grew with more growth from the cells transformed with pSB1C3-chromoproteins than the equivalent pSB3C5 (the low copy number plasmid). The transformed cells were then used to make master plates for the 1C3 plasmid but not 3C5 due to low growth. The 3C5 transformed cells were left to grow in the incubator for another 24 hours.

August 11

After the extra incubation period, the pSB3C5 transformed TOP10 cells had grown to roughly the same level as the pSB1C3 transformed cells. Thus, a master plate was made for the 3C5 transformed cells and incubated overnight at 37℃.

The 1C3 transformed cells were then used for cPCRing of all the chromoprotein constructs. On the confirmation gel there were sadly no bands apart from the ladder.

August 12
The cPCR from yesterday was repeated but using colonies from 3C5-Chromoprotein transformed TOP10. On the verification gel there were no bands for any of the blue transformants but the pink transformants had consistent bands at 1.1kb.

August 15
The protocol to sporulate *Bacillus* was started. Overnights were made using *Bacillus* from the untransformed master plate.

The cPCR from August 11 and 12 was repeated. The products were run again on a gel to confirm PCR.

pSB1C3 transformed TOP10		pSB3C5 transformed TOP10	
Pink Chromoprotein	Blue Chromoprotein	Pink Chromoprotein	Blue Chromoprotein
Consistent band at 800 base pairs for all three types of promoters	Consistent band at 800 base pairs for all three types of promoters	Consistent band at 800 base pairs for all three types of promoters	Consistent band at 800 base pairs for all three types of promoters

August 16
pSB3C5 plasmids minipreped from the overnight cultures from August 15. Because the cultures grew less than expected, the tubes were left in the incubator till later in the afternoon. The minipreps were done using the Omega Biotek E.Z.N.A plasmid mini kit and followed the protocol+Rachelle's notes. 10ml of culture was used to offset the generally low yields from *Bacillus* and the low copy number plasmid. The yields that were obtained were low, ranging from 16.0-28.9ng/ul, with a A260/280 values of 1.88-2.19.

The plasmids containing the chromoproteins were minipred with the homebrew kit, using the overnights made from the successful cPCRs from August 14.

Plasmid	Blue or Pink?	Promoter+Colony#	Yield (ng/ul)	A260/280
pSB1C3	Blue	Pveg 6	51.9	1.86
pSB1C3	Blue	PsspB 5	63.5	1.98
pSB1C3	Blue	PsspB 6	50.9	1.88
pSB1C3	Blue	BBa_K780003 5	77.5	1.92

pSB1C3	Blue	BBa_K780003 6	24.9	1.51
pSB1C3	Pink	Pveg 5	43.7	1.95
pSB1C3	Pink	Pveg 6	172.3	1.95
pSB1C3	Pink	PsspB 5	63.6	1.94
pSB1C3	Pink	PsspB 6	29.1	1.72
pSB1C3	Pink	BBa_K780003 5	44.6	1.96
pSB1C3	Pink	BBa_K780003 6	293.5	1.91
pSB3C5	Blue	BBa_K780003 3	73.5	1.96
pSB3C5	Blue	BBa_K780003 4	41.9	1.89
pSB3C5	Blue	PsspB 3	60.1	1.99
pSB3C5	Blue	PsspB 4	67.1	1.94
pSB3C5	Blue	Pveg 3	261.6	2.00
pSB3C5	Blue	Pveg 4	27.5	1.94
pSB3C5	Pink	Pveg 1	41.3	1.95
pSB3C5	Pink	Pveg 2	19.4	1.97
pSB3C5	Pink	Psspb 1	20.9	1.66
pSB3C5	Pink	BBa_K780003 2	33.5	1.89

Inserts of *ComK* with *AmyE* homology regions inside the prefix and suffix (new) and with *AmyE* homology regions outside the prefix and suffix (old) were digested with EcoRI and SpeI following the protocol on page 5 of Rachelle's lab book. Plasmids pSB1C3-RFP, pSB3C5-I52001 and pSG1154 were also digested with EcoRI and SpeI following the same protocol. The old *ComK* insert will be ligated into pSG1154 for future chromosomal integration in *B. subtilis* as well as pSB1C3 for amplification, storage and registry submission. The new *ComK* insert will be ligated into pSB3C5 to mimic the effects of multiple sites of chromosomal integration (as it is a low copy number plasmid, as well as pSB1C3 for amplification, storage and registry submission.

The pSB1C3, pSB3C5 and pSG1154 vectors were treated with Antarctic phosphatase as per the protocol on page 5 of Rachelle's lab book to inhibit the plasmids from self-ligation. The following was added to each tube of digested vector (4 tubes total, 1 pSB3c5, 1 pSG1154 and 2 pSB1C3):

5 uL 10X Antarctic phosphatase buffer

9 uL ddH2O
1 uL Antarctic phosphatase

The following combination of *ComK* insert to vector was performed following the ligation protocol

Old *ComK* (without *AmyE*) into pSB1C3
Old *ComK* (without *AmyE*) into pSG1154
New *ComK* (with *AmyE*) into pSB1C3
New *ComK* (with *AmyE*) into pSB3C5

The products were stored at -20°C.

Since we thought our competent cell preparations weren't very good, we did one quality control transformation with a randomly selected aliquot. One aliquot of competent TOP10 was transformed with 10ul pSB1C3-RFP at 40.3ng/ul as per the standard transformation protocol for *E. coli*. The transformants were plated on Chlor plates and incubated overnight at 37℃.

Note: there was initially no growth but after a replating and another day of incubation, RFP expressing cells were observed on the plates but at a very low transformation efficiency

August 17

Samples for sequencing we prepared using 160-200ng of template DNA. The template DNA are all four of the BBI constructs. 1 ul of either forward or reverse primer was used in each tube and the remaining volume to 12ul was filled with ddH_2O. In total there were eight reactions, four forward and backwards.

More work was done on sporulating cells. The overnight cultures were diluted 1:200 in the 2xSG media and incubated at 37℃ and 200rpm. The culture was checked for spores approximately every 24 hours using a gram stain. The protocol was the same one used by the 2014 Calgary iGEM team. The protocol can be found on Open Wet Ware.

Minipreps of the chromoprotein plasmids were redone but only for the ones that had a low yield. The new miniprep did not improve on the previous low yields from August 16.
However the well preped samples from August 16 were prepared and sent down for sequencing.

August 18

To check for the presence of spores in culture, a gram stain was performed. Spores will not stain and remain colourless as the spores are impermeable to any of the stains while the unsporulated *Bacillus* will stain purple (gram positive). Under the microscope there were very few spores present ~<10%.

4 aliquots of TOP10 cells were transformed with the ligated products from August 16 following the transformation protocol. 10ul of ligation products were used in the following transformations:

pSB1c3 - old *ComK*	plated on Chlor	
pSG1154 - old *ComK*	plated on Amp	
pSB1c3 - new *ComK*	plated on Chlor	
pSB3c5 - new *ComK*	plated on Chlor	
pSB1c3 - RFP	plated on Chlor	positive control

August 19

The second gram stain of the developing spores revealed greater sporulation than August 18 but there was not a clear majority of spores relative to vegetative cells.

The *Bacillus* that was streaked the previous night was then transformed with the plasmids (pSB1C3 and pSB3C5) containing the chromoproteins and their respective promoters.
The TOP10 transformed on August 18 failed to grow on any of the plates. Those plates were left in the incubator while fresh plates were spread with transformed cells from August 18. And because we think there are issues with the cells that were made competent, an untransformed aliquot of cells from the glycerol stocks were streaked and incubated at 37°C.

August 20
Still no growth on either set of plates...we think it's the competent cells, they were made from an old batch of untransformed *E. coli* TOP10 and also took a really long time to grow to an OD600 of 0.4 during competency protocol.

Gram staining of the *Bacillus culture* revealed that the vast majority of the cells had sporulated. This was seen under the microscope as colourless coccoid objects. Spores (10mL) were removed from the original culture and spun down for 2 minutes at 14k rpm. The spores could be differentiated from vegetative cells by the dark brown colour of the spores. Isolated spores were stored in fresh ddH_2O at -20°C.

> **Note**: This was all that was done with the spores as we had to focus on the expression of BBI over an sporulation experiments. We figured that we could use the 2014 UCalgary Team data if necessary.

August 21
New, fresh cells were made competent as per the protocol on page 2 of Rachelle's lab book. During resuspension (step 4) and aliquotting (step 6), the procedure was performed in the cold room at 4°C. Cold pipette tips and aliquot tubes were used throughout the protocol. Step 6 was performed (samples were placed on ice for 30 min) before storing aliquots at -80°C.

August 22
The competent cells that were made yesterday were tested by transforming 3 aliquots of freshly-made competence cells with the following samples of DNA (following the normal transformation protocol.

Tube 1: 7.4 uL of pSB1c3-RFP (120 ng/uL)
Tube 2: 1 uL of pSB1c3-RFP (0.2 ng/uL)
Tube 3: nothing (negative control)

After adding DNA, the aliquots were left to sit on ice for 1 hour (instead of 30 minutes) to allow for DNA to enter each cell.

After transforming, 100 uL of sample was plated on Chlor plates and left to incubate at 37°C O/N.

The *Bacillus* that was transformed yesterday grew on the LB-agar plates.

August 23
Growth was observed on both RFP transformed plates and none on the negative control plate (**our cells are competent!**)

4 aliquots of competent TOP10 cells were transformed with the following ligated *ComK* products as per the normal transformation protocol.

Tube 1: pSC1c3-*ComK* without homology (11.25 uL) plated on Chlor
Tube 2: pSG1154-*ComK* without homology (10.5 uL) plated on Amp
Tube 3: pSB1c3-*ComK* with *AmyE* homology (10.4 uL) plated on Chlor
Tube 4: pSB3c5-*ComK* with *AmyE* homology (11.0 uL) plated on Chlor

After adding DNA to the competent cell aliquots, the cells were left on ice for 1 hour instead of 30 minutes. 100 uL of each samples was plated on the appropriate plate instead of 50 uL. Cells were left to incubate at 37°C O/N.

pSB1c3-RFP was prepped from the O/N prepared yesterday by following the homebrew protocol which gave the following yields.

Tube 1 of pSB1c3-RFP: 60.9 ng/uL A260/280: 2.11
Tube 2 of pSB1c3-RFP: 56.3 ng/uL A260/280: 1.54
Tube 3 of pSB1c3-RFP: 58.9 ng/uL A260/280: 1.96
Tube 4 of pSB1c3-RFP: 58.2 ng/uL A260/280: 2.35

The prepped pSB1c3-RFP plasmids were singly and doubly digested following Rai's protocol.

Plasmid	Single Digest	Double Digest
Tube 1: pSB1c3-RFP (60.9 ng/uL	EcoRI	EcoRI + PstI
Tube 2: pSB1c3-RFP (56.3 ng/uL)	EcoRI	EcoRI + PstI
Tube 3 of pSB1c3-RFP	EcoRI	EcoRI + PstI
Tube 4: pSB1c3-RFP (58.2 ng/uL)	EcoRI	EcoRI + PstI

The 8 digests were run on a 1% agarose gel using an Amresco 1 Kb DNA ladder

Lane	Contents	Expected Size (bp)	Observed Size (bp)
1	LAdder		
2	pSB1c3-RFP #1, single digest	3100	3100
3	pSB1c3-RFP #1, double digest	1000, 2100	Nothing
4	pSB1c3-RFP #2, single digest	3100	3100
5	pSB1c3-RFP #2, double digest	1000, 2100	Nothing
6	pSB1c3-RFP #3, single digest	3100	3100, 5000
7	pSB1c3-RFP #3, double digest	1000, 2100	1000, 2100, 2500

8	pSB1c3-RFP #4, single digest	3100	3100, 5000
9	pSB1c3-RFP #4, double digest	1000, 2100	1000, 2100, 2500

Based on the gel, we have to redo the minpreps. There was obviously something wrong as the 5000bp was 2000bp larger than any of plasmids miniprepped!

August 25

Following the *Bacillus* transformation protocol from Dr. Wong, *Bacillus* was transformed with the 3 different promoter+chromoprotein constructs in both pSB1C3 and 3C5 vectors. The transformed cells were streaked onto LB-Chlorplates and incubated overnight at 37°C.

The *ComK* inserts with and without *AmyE* homology were PCR amplified using the Prefix-F and Suffix-R primers and the PCR protocol found on page 6 of Rachelle's lab book. The products were run on a 1 agarose gel with a 1 KB PLus DNA Ladder.

Lane	Contents	Expected Size (bp)	Observed Size (bp)
1	amplified *ComK* without homology		
2	amplified *ComK* with *AmyE* homology	970	970
3	DNA ladder	1200	1200

The inserts were successfully amplified, hallelujah! They were nanodropped to give the following yields:

Amplified *ComK* without homology 85 ng/uL
Amplified *ComK* with *AmyE* homology 60 ng/uL

4 vector tubes and 3 insert tubes were digested and treated with fast Antarctic Phosphatase following standard digestion protocol on page 5 of Rachelle's lab book:

<u>pSB1c3 tube #1</u>
10 uL pSB1c3 (50 ng/uL)
3 uL 10X Cutsmart buffer
1 uL XbaI
1 uL SpeI
1 uL Fast Ap
In 30 uL total with ddH2O

<u>pSB1c3 tube #2</u>
10 uL pSB1c3 (50 ng/uL)
3 uL 10X Cutsmart buffer
1 uL XbaI
1 uL SpeI
1 uL Fast Ap
in 30 uL total with ddH2O

<u>pSG1154 tube</u>
5 uL pSG1154 (200 ng/uL)
3 uL 10X Cutsmart
1 uL EcoRI

<u>pAB3c5 tube</u>
1 uL pSB3c5 (545 ng/uL)
3 uL 10X Cutsmart
1 uL XbaI

1 uL SpeI
1 uL Fast Ap
In 30 uL total with ddH2O

1 uL SpeI
1 uL Fast Ap
in 30 uL total with ddH2O

ComK without homology tube #1
10 uL *ComK* without homology (85 ng/uL)
2 uL 10X Cutsmart
1 uL XbaI
1 uL SpeI
In 20 uL with ddH2O

ComK without homology tube #2
10 uL *ComK* without homology (85 ng/uL)
2 uL 10X Cutsmart
1 uL EcoRI
1 uL SpeI
in 20 uL with ddH2O

ComK with *AmyE* Homology tube
13 uL *ComK* with *AmyE* Homology (60 ng/uL)
2 uL 10X Cutsmart
1 uL XbaI
1 uL SpeI
In 20 uL with ddH2O

All 7 tubes were left to digest at 37°C for 2 h before the restriction enzymes were heatkilled at 80°C for 20 minutes. Products were stored at 4°C O/N

August 26
The plasmid were miniprepped from the overnight cultures of transformed *Bacillus* from August 25. The plasmids were then sent to be sequenced. Three constructs were sequenced: Pveg+Blue chromoprotein in pSB3C5, PsspB+Pink in pSB1C3, and BBa_K780003+Pink in 3C5.

ComK was ligated into 3 different vectors in the following procedure. 4 ligation tubes containing the following insert and vector combinations were ligated for 6 hours at room temperature:

1. pSB1c3-*ComK* without homology
3.5 uL pSB1c3 digested with XbaI + SpeI (16.7 ng/uL)
7 uL *ComK* without homology digested with XbaI + SpeI (42.5 ng/uL)
2 uL 10X ligase buffer
0.5 uL T4 DNA ligase
In 20 uL total ddH2O

2. pSG1154-*ComK* without homology
1.5 uL pSG1154 digested with EcoRI + SpeI (16.7 ng/uL)
10 uL *ComK* without homology digested with EcoRI + SpeI (42.5 ng/uL)
2 uL 10X ligase buffer
0.5 uL T4 DNA ligase
In 20 uL total ddH2O

3. pSB1c3-*ComK* with *AmyE* homology
3.5 uL pSB1c3 digested with XbaI + SpeI (16.7 ng/uL)
7 uL *ComK* with *AmyE* homology digested with XbaI + SpeI (39 ng/uL)
2 uL 10X ligase buffer
0.5 uL T4DNA ligase

In 20 uL total ddH2O

4. pSB3c5-*ComK* with *AmyE* homology
3 uL pSB3c5 digested with XbaI + SpeI (31.3 ng/uL)
4.5 uL *ComK* with *AmyE* homology digested with XbaI + SpeI (39 ng/uL)
2 uL 10X ligase buffer
0.5 uL T4 DNA ligase
In 20 uL total ddH2O

The ligated *ComK* products from above (tubes 1-4) and a pSB1c3-YFP plasmid from the 2016 iGEM registry kit (plate 4, well 2M) were used to transform 5 aliquots of competent *E. coli* TOP10 cells following the protocol on page 2 of Rachelle's lab book. 100 uL of each sample was plated on the appropriate plate (Chlor for pSB1c3/3c5 plasmids, Amp for pSG1154 plasmid) and left to incubate O/N at 37°C.

August 29
Sequencing results came back for the chromoprotein constructs and it was discovered that the sequences aligned perfectly to RFP. The sequences for when the chromoproteins were transformed into *E. coli* were checked as well and we discovered that, they too, were RFP.

September 6
An overnight culture (5mL) of *Bacillus* transformed with BBI 1 was started and left to incubate throughout the day at 37℃ and 200rpm. While the culture was incubating, 1L of plain LB broth was prepared and autoclaved in a 2L Erlenmeyer flask. At 5pm, the media was inoculated with the 5mL of *Bacillus* culture. The 1L culture was left to grow overnight at 37℃ and 200 rpm.

September 7
800mL of overnight culture was taken out of the 2L flask and transferred to several 50mL falcon tubes. Then through a series of spin downs and suspensions, the *Bacillus* was concentrated into one mega pellet in a single falcon tube. The mega pellet was then resuspended in 10mL of ddH_2O.

The resuspended cells were then sonicated in order to rupture the cell membranes. This was necessary in order to measure the amount of BBI produced by the cells but not secreted. The sonicator was set to pulse for 30 seconds followed by 30 second pause. This cycle repeated 5 times. The amplitude of the pulse was 30% of maximum power. We were given a demonstration in how to operate the sonicator from Ms. Linda Lee who was in charge of it. The broken up cells were then aliquoted into 2mL tubes and spun down for 10 minutes at 14k rpm. The samples were stored at -20℃ overnight.

September 8
Samples of the cell lysate and media were diluted 1:10, 1:100, and 1:1000 and delivered to Dan for analysis with mass spectrometry. There were no results unfortunately.

September 9-October 14
Attempting to clone our assorted parts into the BioBrick backbone (pSB1C3). This has had limited success. We discovered realized that we never had BBI in any plasmid and even worse, all of our stocks had been degraded. Forcing us to get more from IDT. At the current point in time the following parts have been parts have been cloned into the BioBrick backbone.

CONSIDERATIONS

What we have to determine:

WEDNESDAY, MAY 4TH

- How effectively diffusivity works
- Scaling:
    - Model and prototype
    - Measurements
- Layers:
    - Kill switch in backing layer and bottom layer
    - Break controller
- Know how long proteins would stay in the body

FRIDAY, MAY 6TH

- Habitation:
    - Reservoir/chamber
    - Control temperature
    - Oxygen permeability
    - Specific parts of the body (heat, placement)
    - Delivery of nutrients - snapping mechanism?
    - Modelling - in different conditions, see the growth rate
- Entire Device:
    - Design
    - Materials - contaminants
    - Eg) rate controlling membranes, diffusion of peptides
- Interface:
    - Needle - size, density, material, design
    - Compatibility - initiation
    - Site of application
    - Disinfect prior to use, etc.

FRIDAY, MAY 20TH

- General
    - How to apply to body?
    - How to discard after use (i.e. when we take it out, won't the media start to leak?)
- Microneedles/Drug Reservoir
    - How to deliver drug through microneedle? (frozen, snap mechanism, isotonic?)
    - Hollow needle location → side or straight down?
    - What to test? (strain, stress, flow rate, diffusivity, etc.)
    - How to construct everything on a small scale?
    - Can we build a microneedle based on our own dimensions (i.e. can we customize the microneedle)? Or are the dimensions preset because we are ordering them in?
    - Backing layer → is that included in the microneedle or do we need to attach it on?
    - Actually getting microneedles
    - What is in the drug reservoir? (bacteria, nutrients (LB = nutrients))
    - Can we build on a microscopic scale? If so, how?
    - What is the media that is in the microneedle?
    - How do traditional microneedles work?
    - Snapping mechanism
- Size-controlling membrane

    - How big should the pores be? (0.1 micrometer or smaller… 0.1 = size before bacteria can go through) → what else other than peptides will go into the body? Will they be harmful?
    - What material should it be made of? What else do we need to consider for the membrane (flexibility, etc.)?
    - How do we get it? (3M)?
    - Can we cut it to get it in a certain size?

WEDNESDAY, MAY 25TH

When researching materials, consider the following:

- cost
- environmentally friendly
- the “biological aspects” (e.g. gas/oxygen permeability, elasticity permeable)
- how can we get the material (local, international)
- think about how can we use the material to assemble the device (e.g. if we need silicon for the device for example, is it easily machined?)

TUESDAY, MAY 31ST

****The patch has to be **transparent** so that the indicator can be noticeable

MONDAY, JUNE 6TH

- How to make the patch **long term**
    - Look at disposable
    - Space the package would take
    - Shelf life, how long would it take for the patch contents to "expire”
- Think about the worse scenarios that we could get
    - Then we can start thinking about alternatives
    - 100 mL vs. 10 mL
    - Packets being unintentionally popped
- Actually have a plan, but it is also important to know how everything works out

DESIGNS

MONDAY, MAY 9TH

DESIGN OF MICRONEEDLES:

- Hollow cylindrical microneedle with conical tip
- Enough strength to withstand bending and axial forces
- Pressure uniform in main cavity of needle
- Velocity constant in cavity; increase in outlet
- Flow rate controlled by applied pressure and diameter of hole
- For different materials - strength and deformation compared

Design 1:

- The microneedle - from the machine shop
- Semipermeable membrane - use a filter (different grades of filter paper)
- Bacteria?
- A top layer (to the bacteria)

Design 2:

Part 1: MICRONEEDLE

- The microneedle - from machine shop
- Semipermeable membrane - use a filter (use different grades of filter paper)

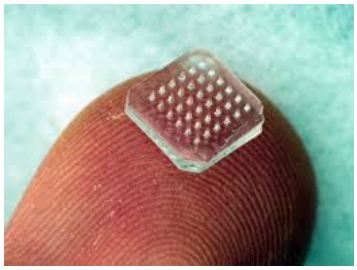
Part 2: CAPSULE WITH BACTERIA

- Capsule with bacteria - the capsule can be put in the fridge; after, it would be inserted into the microneedle and then using a snapping mechanism/force to break the capsule which starts the process and peptides go through the semipermeable membrane

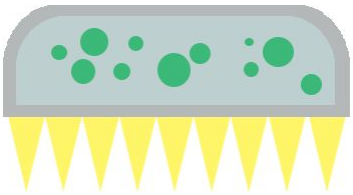
TUESDAY, MAY 17TH

Device Prototypes:

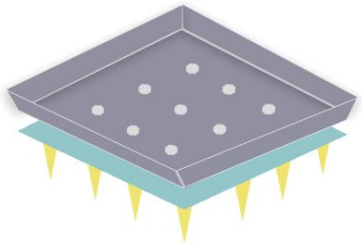
- *Major idea #1: Bacteria, rich media (LB), membrane all in top of drug reservoir*



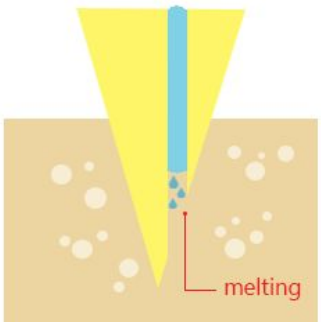
- Variations of Major idea #1
  - Patch on top of microneedle array (patch is directly on top of patch)



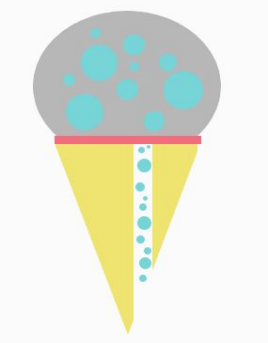
  - Drug reservoir right on top of needles (i.e. no patch) and size controlling membrane
    - Problems: How can we actually put in things in this small drug reservoir?



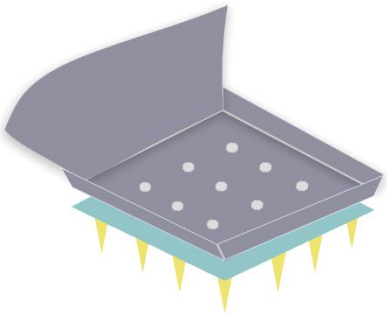
- Drug reservoir right on top of needles – media is initially frozen and then melted after application to body due to body heat
  - Problems: can't make the media frozen because expensive (buy refrigerator)
  - Frozen media will also expand like water, so when it melts it may not come into contact with spores



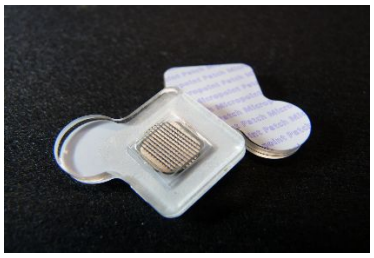
- 1 packet containing the bacteria in the drug reservoir → snap the packet to release bacteria spore
  - Problems: How can we actually put spores in small packets and then put them in the small drug reservoir? How can we break those packets?



- Introduction of a gel (on a "lid"/backing layer that is lowered onto the top of the microneedle array/drug reservoir) OR rate controlling membrane (hence we have a rate and size controlling membrane)
  - Problems: How do we put a lid on the top of the microneedle array? How do we stick the gel on the lid? If the gel falls, won't it block the size controlling membrane? How do we put a rate controlling membrane in the small microneedle array?



- *Major idea #2: Leave microneedle as it is; have larger patch that contains 2 chambers to separate LB and spores*



- backing material (what the block is made of)
- "valve"
- size controlling membrane
- adhesive layer
- microneedles

Constraints:

- 70 degrees C to activate spores
- Oxygen transmission: available (80-100 cc/m2/24h)

Dr. Dalton's Microneedles

- **Problem 1**: Geometry of microneedles
  - TIP:
    - Insertion force can be independent of the wall thickness
    - Fracture force increases with increasing wall thickness and increases with wall angle, also independent of tip radius
    - Side opened microneedles instead of standard tip microneedles – prevents tissue clog during insertion
    - High needle density can increase fluid flow rate
    - Sharper needles require less force for insertion, but has reduce needle tip strength
    - Microneedle needs to be 10-15 um but shorter than 50-100 um to avoid pain
    - Best option: small tip radius, large wall thickness
  - BODY:
    - Cylinder: eliminate stress concentrations, stronger needle structure, better self-adhesion
- **Problem 2**: Material for microneedles
  - Single crystalline silicon: high resistance to bending, can be fragile
  - Metal – greater strength, but thin metals are soft
- **Problem 3:** Application/removal
  - Application: Elastic nature of skin creates possibility of non-uniform contact of the array with the skin - an issue for correctly metering dosages
    - Has to find out which area of the skin where the least strain happens
  - Removal: Leakage when microneedle patch is removed

- Development of microvalves within microneedles = passive system

Reference: Ashraf, M., Tayyaba, S., Nisar, A., Afzulpurkar, N., Bodhale, D., & Lomas, T. et al. (2010). Design, Fabrication and Analysis of Silicon Hollow Microneedles for Transdermal Drug Delivery System for Treatment of Hemodynamic Dysfunctions. *Cardiovascular Engineering*, *10*(3), 91-108. http://dx.doi.org/10.1007/s10558-010-9100-5

MONDAY, MAY 9TH

FUTURE DESIGN TESTING:

- Test the proposed design with skin (animal skin?)
- Change diameter of hole - how much more different is the flow
- Heat of finger - enough to stimulate it?
- Diffusion works?
- Failure load (at what pressure does the needle break?)

STORAGE IDEA 1: FREEZE DRYING

- Order in microneedles > put in freezer > at temperature of freeze dry
    - If microneedles survive in the fridge, we should also test if diffusivity still works
    - If diffusion did not work, we have to look at pumps
- Membrane/Filter test: does the drug reservoir/peptide actually go through the filter? Does it prevent the bacteria from going through?
- Diffusion test: To test whether diffusion will actually work and what other problems we will face with diffusion (e.g. bacteria secretes peptides which go through the semipermeable membrane… but what about the media that goes through the membrane?
- Making changes to the design
    - Is the drug reservoir actually contained properly in the microneedle?

- Bacteria test

MONDAY, MAY 16TH

EXPERIMENT TO SOMEHOW TEST THE IDEA:

Objective:

Tested the level of frozen H2O in microneedle as it melts into a cup of H2O (l)

Materials:

- 200 uL pipet tip
- Distilled H2O
- 200 uL pipetter
- Freezer
- 300 uL H2O
- Parafilm/tape
- Black sharpie
- Test tube rack

Procedure:

1. We used a pipet to take in 150 uL of distilled water in a pipet tip and taped the bottom with parafilm/tape. We marked the water level with a black sharpie.
2. Put the pipet tip in freezer and let the H2O freeze (10:50 am - after lunch).

3. Put pipet tip (just the tip area) into test tube of 300 uL (2x the volume of water in the needle). Cap the top with parafilm.
4. Overtime, see whether amount of H2O (l) goes back to original height.

Results/Observation:
After putting the frozen water in the distilled H2O (l) test tube (at normal tap temperature), we put parafilm on top to mimic the microneedle being capped.

- H2O melts extremely fast (this is without adding body heat) - melted within 1-2 minutes
- In actual microneedle, H2O (s) will melt very fast due to body heat. Also, silicon vs. plastic tube
- After melting we noticed H2O (l) level in needle is at a constant level (significantly lower than initial amount). Eg) doesn't go back to original level
- H2O did expand, but not too significantly from initial observation, we think it won't affect/damage the needle tip

MONDAY, MAY 9TH

IDEA 2: POPPING IDEA

- Instead of pump, have a chamber with cells have a positive pressure already within it - have it sealed
- Capsule - look into the 2014 team
- Thinking about having water in the capsule, keep water in capsule, break it and everything gets hydrated
- Coating the microneedles with palmitic acid, we don't need to create another compartment
- For medical uses, need only single administration, would we need the bacteria there? Could dissolving microneedles work?

MONDAY, JUNE 6TH

Tentative Materials for Our Patch

Part of the patch	**Material**	**Pros**	**Cons**
Backing Layer Purpose: provides structural support and protects the middle adhesive layer from the environment	**3M CoTran™ 9722 Backing Polyethylene Monolayer Film** Alternative: (2nd best out of 3) 3M CoTran™ 9719 Backing Polyethylene Monolayer Film	Out of the three films 3M offers, the qualities that we thought would be beneficial are: ● Elongation = for movement of patient, has to 600% elongation, highest of the three ● MVTR = this has the lowest amount out of all 3 Translucent Breathable Printable Can be directly laminated to adhesives Heat Sealable (PE) Designed to resist excipient and drug uptake	We are not sure how much oxygen the bacteria would be using. We are not sure if 6400 $cc/m^2/day$ is enough for oxygen transmission. We do not know if this would turn cloudy after a period of time as currently existing patches with clear backing undergo the same issue.

Adhesive Layer Also note that thicker adhesive layer also results in severe cold flow during storage in the pouch, and higher affinity for lint and dirt to adhere to the edge of the patch during wear.	pdf of Duro-Tak Transdermal Adhesives		
Release Liner	3M Scotchpak 1022 3M Scotchpak 9741 3M Scotchpak 9742 3M Scotchpak 9744 3M Scotchpak 9755	• Good for release with silicon skin contact adhesives, acrylate, PIB and rubber based PSA • Excellent chemical stability	
Size-controlling membrane	tentative		

IMPORTANT NOTES

MONDAY, JUNE 20TH

- Emailed 3M for products, will talk to them tomorrow if they haven't replied.
    - Got a reply from 3M. Will need to call them later to ask for products.
- Emailed Dow Corning for adhesives
- Emailed Dr. Nezhad, an Electrical Engineering prof whose research is focused on microfluidics, for meeting
- Researched about different assays for material testing

MONDAY, JUNE 27TH

- Contacted 3M again, they are sending us the samples (YES). They should be here by the end of next week.
- Dow Corning will send the adhesives on 29th of June
- Today the device team focused on the math model for diffusion as weird numbers for the initial amount of peptides that needs to be produced have been calculated. Noshin found a paper with a MATLAB code that can be used to calculate the diffusion across a membrane using Fick's second law of diffusion. An email was sent out to Dr. Nygren to get his opinion on the code and see if we are heading in the right direction
- UPDATE: We meet with Dr. Nygren Wednesday to go over the mathematical model developed. He suggested in his email that the equation is applicable in principle, but the situation is a little different because the membrane and skin surface are probably the main diffusion barriers and the diffusion constant is (very) different at those barriers compared to everywhere else. He also suggest we create a numerical method rather than doing it analytically.

TUESDAY, JUNE 28TH

- The device group continued to work on both learning Solidworks to build the graphical model as well as the mathematical model of the diffusion of BBI through the patch, skin and into the bloodstream.

- From our results we calculated that we need 2 g of peptide being produced in the patch. This however is not feasible and needs to be reworked.

Wednesday, June 29th

- Protocols Tiff and Dave talked about with Dan
    - Test how much media go through and if peptides go through; safety mechanism
    - Can we make dye as a qualitative or quantitative aspect of the assay?
    - How do we confirm peptides make it through?
    - Critical: make sure cells stay where they are, and peptides go through
    - There may be a big difference in terms of the diffusion constants depending on the volume, barriers, material
    - Dr. Nygren's suggestions
        - DRAW
        - Volumes separated by membranes
        - Faster production = would reach steady state at some point as too much BBI might stop bacteria from producing
        - Work equations out that he derived; make sure units are consistent
        - Degradation - might be in the bloodstream, not before the skin
        - Diffusion coefficients: testing would be necessary for peptide flow through membrane; for skin, could probably find from literature; or ask yourself, can we just find the diffusion coefficients rather than testing bunch of membranes?
        - In our case diffusion is too fast that equilibrium is achievable.
        - Make sure to state assumptions and a way to justify it.

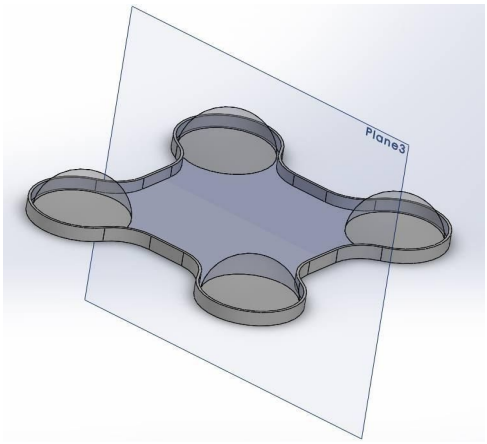
        - MATLAB: solving ODEs; recall ENGG 407 lecture

Tuesday, July 5th

- The rest of the team continued to work on the diffusion model based on Dr. Nygren's suggestions. Based on Noshin's work, there were too many unknowns that we could solve for if we decided to solve the equation as a function of time. Therefore we went back to Fick's first law where the diffusion was a function of concentration over area. This however had unknowns such as protein solubility that we weren't sure of
- Adhesive? Gave the green light that the assays are doable
    - Backing Layer? Gave a similar opinion that the assays are doable
    - Membrane? REASONABLE
    - Quantifying about how much peptide go through
    - Use of other peptides? (Since we want to quantify diffusion, maybe we could use something that is cheaper)
    - TRICKIEST: finding how much went through
    - Glucagon, oxytocin (20 - 30 amino acids long)

Wednesday, July 13th

- Contacted Dr. Nezhad about manufacturing patches, asked for resources.
- Contact Dr. Ingalls from Waterloo.
- Update: Waterloo is open for collaboration.
- In terms of modelling:
    - Had our equations sent to Brian Ingalls for checking.
- Christine developed the patch using SolidWorks! See below:



THURSDAY, JULY 14TH

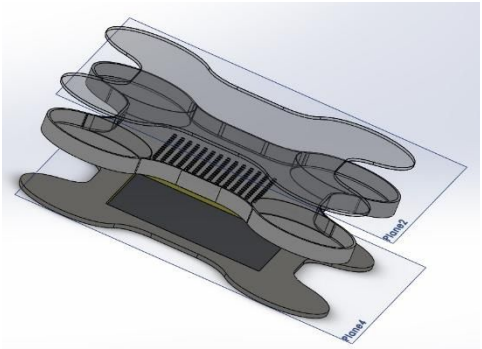
- Meeting with Dr. Sundararaj (UT) next thursday the 21st at CCIT 320 at 1 pm for manufacturing.
- Finished the analytical diffusion model across stratum corneum. See Device folder >> Patch Modelling >> open the only document there >> See Stratum corneum under Layer by Layer heading.
- Tiffany finished her initial models for the bottom layer of the patch
    - Optimized the dimensions to hold 10 mL in the main area, and either 0.5 and 1 mL of media in the pockets
    - Sent the models into 3D printing services at TFDL <3
    - Will hear back in a couple of business days about the progress

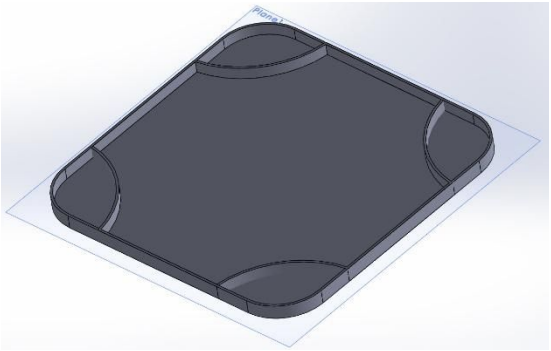
FRIDAY, JULY 15TH

Team Meeting

- Nelly has finished the first part of the analytical model for the stratum corneum. She will continue working on the next two parts of the analytical model for the next week
- Dave has been researching ways to manufacture to create the patch. For most industries including 3M they will only manufacture for large scale industry
- As a result Dave is focussing on how to manufacture our patch ourselves or with some professors
- Nelly will help out with Dave with this by researching into the adhesive and how to attach it
- Both Nelly and David have contacted professors for help in manufacturing the patch. We have a meeting with Dr. UT next Thursday at 1:00 pm with David, Nelly and Tiff
- If worse comes to worse and no one can help us, we are visiting the machine shop to see if anyone can help us
- Christine and Tiff have finished their prototypes. Tiff has sent hers to get 3D printed and will hear back in a couple of business days about the progress
- Christine and Tiff will start David's assay next week and run triplicates throughout the week to see if the patch growth curves matches the ones under optimal conditions already done in the lab
- Discussed with Nishi what needs to be done for mouse trials
    - There are three trials being run: 6 mice for a positive and negative control, 6 mice with patches with BBI dissolved in DMSO, and 6 mice with patches with cell culture
    - The patch is 1 cm X 1 cm X 0.2 cm
    - The patches need to be completed Monday prior the experiments begin the following Monday
- Alina also messaged back Tiff to see how things were going
    - She will be escorting an astronaut from the Apollo 11 mission during a science fair and so we can send her questions we can ask the astronauts that will be there

- Tiff will create a document for questions to send to Alina. Focus the questions on either radiation in space or their lifestyles in space
- As well she said she will try to help us with our model. She said to message her as a group if we have questions about the diffusion model. Currently she is working on her own MATLAB project and she said could help us with this aspect specifically





Tuesday, July 19th

- This is what Nelly did for the whole day: Check this out. She tried to organize all the models she had on file into a document. Hopefully it is easier to navigate in this format. She also uploaded all the codes, functions, scripts etc. She uploaded it in a folder under Patch Modelling.
- Followed up with Dr. Nezhad because he has not responded for 6 days.
- Finished first draft of the powerpoint about our project. Check *presentation under *device for it.
- Tiffany talked to Dr. Mayi concerning optimizing the diffusion assay
    - Instead of an eight hour interval of taking the sample, we will run the project over a seven day period, taking samples at 24 hour period to mimic the patch.
    - As well, we are now measuring the the optical densities over a spectrum as no literature points to a wavelength for saline solution/distilled water

Wednesday, July 20th

- Tiffany continued working on the diffusion assay and got initial results to determine the wavelength needed to determine the optical density of saline solution
    - The falcon tubes containing LB, *B. subtiliis* and *E.coli* were removed from the saline solution
    - Saline solution was used as a blank
    - Wavelengths of 260 nm, 300 nm, 600 nm, and 700 nm were used to measure the absorbance of the saline solutions contained in the erlenmeyer flask

Wednesday, July 27th

- Went to the machine shop to see if they can help us manufacture patches for prototype testing.
    - Although they were not super clear about what we wanted initially they figured out the basics of what we needed from them
    - They took care of 3D printing for free for us as we are a student based project and they resolved the issues for us
    - Tiffany is in contact with them and will hear back from them in a couple of days
    - In addition, we learned that the machine shop cannot manufacture the patch. What they do recommend is the easiest way would be to proceed with something similar to the thermoforming method
- About the thermofolding method

    - Suggested we cast a mold that they can help us design using metal. The mold would consist of two parts for the backing layer and the semipermeable membrane
    - We would then heat our materials and lay them onto the molds to conform to their shape. From there we place the molds together and inject our media into the mold and heat seal the entire patch together
    - Unfortunately they can only help us with the mold. They don't have equipment we could use
- We're gonna talk to Dr. Mayi/Nygren before we proceed to see if this is a good idea and how we can proceed about thermoforming.
- We also need to determine experimentally at what temperature we get the two materials to seal together. This has to be determined ourselves
- Tiff will bring hair iron + blow dryer tomorrow to test at what temperature we can heat seal our materials

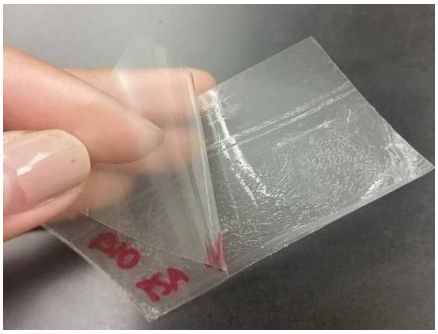
THURSDAY, JULY 28TH

- The device team made prototypes today!
    - We used the flat iron, iron, and blow dryer. Iron worked best. See pic below!

- Nelly worked on her adhesives. She tested BIO-PSA 7-4101 and 7-4301; however, both tests failed. What she did on her first test was she applied a thin film of adhesive on the release liner and let the heptane evaporate. She waited for 20 minutes, but the adhesive dried completely that it lost its adhesive properties. She tried the second time and she waited for heptane to evaporate for 5 minutes. The adhesives still contained heptane. She would run tests again tomorrow for 10 and 15 minutes.

FRIDAY, JULY 29TH

- Had our weekly meeting. See timeline for what we have to accomplish next week.
- David contacted 3M to ask them to create a crude prototype so we have a better idea what the patch should look like.
- Nelly did rounds of her adhesive testing assays. She found that the BIO-PSA 7-4201 will be the best adhesive for our current purposes. BIO PSA 7-4101 dries up quickly as when it was applied, it lost it adhesiveness. BIO PSA 7-4301, on the other hand, dries up very slowly, which also explained why it is less viscous than the other adhesives. This means it is dissolved in more heptane. In the pic below, Nelly was holding onto the release liner, the other layer was our membrane. The adhesive was successfully stuck to the membrane.
    - Recommendations:
        - Use of rolling pin for more uniform adhesive distribution.
        - Wait 1.5 minutes for the adhesive to dry up once spread as film before attaching the membrane.
        - Test on pork skin. Design assays for quantifying amounts of adhesive we need to apply, optimal temperature for drying, etc.
        - More prototypes next week!

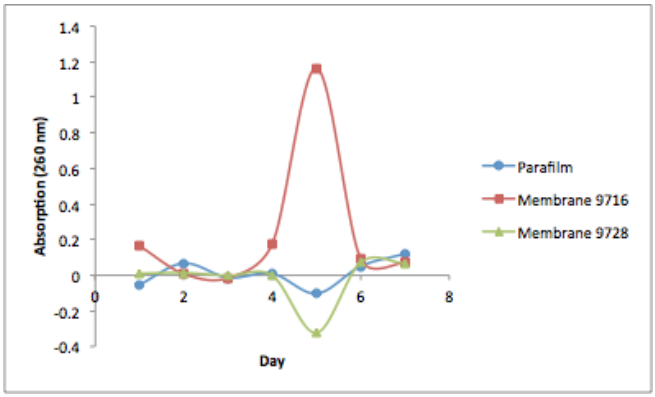


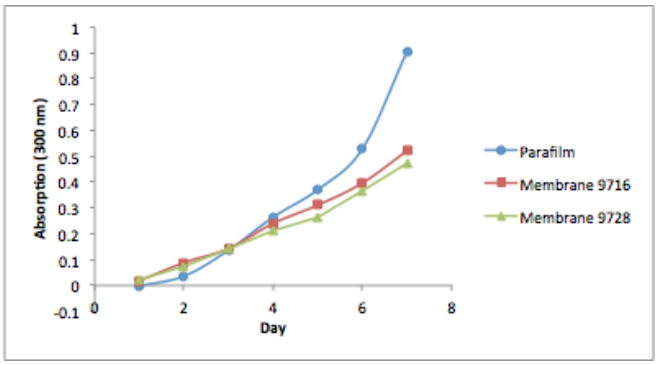
TUESDAY, AUGUST 2ND

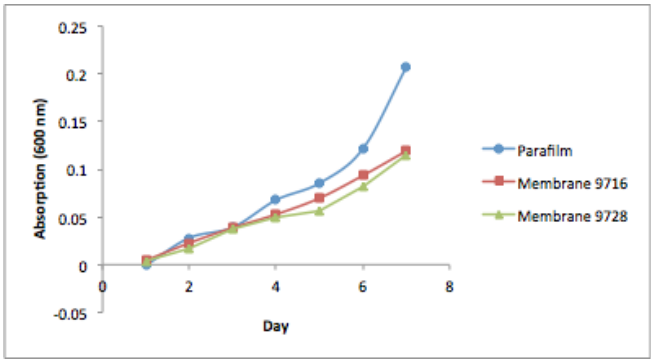
- David finished a detailed word doc outline of the presentation (with some help from Nelly and Tiff <3). Check under Presentation folder and edit anything you'd like. Now pass on to Christine to create powerpoint. Christine started a rough draft of our presentation.
- In the lab
    - Tiff finished the last day of the diffusion assay
    - Nelly and David performed some adhesive experiments. They experienced problems and they are as follow:
        - The adhesive did not stick to the liner completely
        - Uneven distribution of adhesive on liner

WEDNESDAY, AUGUST 3RD

- Christine did the data analysis for the diffusion assay







- Nelly and David are testing adhesive. There were different methods used in doing these tests. They are as follow:

a. Rolling pin test

i. An amount of adhesive was applied to the release liner.
ii. A cut out of EVA membrane was placed onto the adhesive.
iii. Place another layer of the release liner on the membrane to prevent the adhesives from sticking to the rolling pin.
iv. Roll the pin on the layer to distribute adhesive.
v. Let it dry.

b. Film application test

i. An amount of adhesive was applied to the release liner. The adhesive must be applied I a straight line.
i. Spread the adhesive using a popsicle stick to form a thin film.
ii. Wait for a minute before placing a cut out of EVA membrane onto the release liner to dry

b. Two - release - liner - sandwiched - together test

An amount of adhesive was applied to the release liner. The adhesive must be applied in a spiral manner.

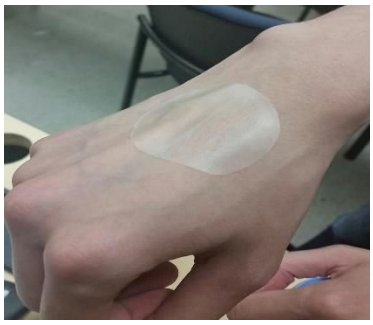
i. Place another layer of the release liner onto the initial liner.
ii. Spread the adhesive using a rolling pin, by pressing, etc. until you see a clear film (meaning, no air bubbles, no accumulated glue anywhere, etc.)
iii. Wait for 20 minutes to let the adhesives settle for a bit.
iv. Peel the liners apart.
v. Place a cutout of the EVA membrane onto the liner.
vi. Let it dry.

Results from the adhesive tests:

a. Rolling pin test: Opposite to what was expected, the adhesives were unevenly distributed. Air bubbles and bumps of glue were present. Some had not completely dried up, forming webs when peeled, some had.

b. Film application test: The adhesives were unevenly distributed.

c. Two - release - liner - sandwiched - together test: The adhesives were unevenly distributed, but this method was the most effective one. See pic below!



- Tiffany also picked up the 3D printed models of our prototype from the machine shop

THURSDAY, AUGUST 4TH

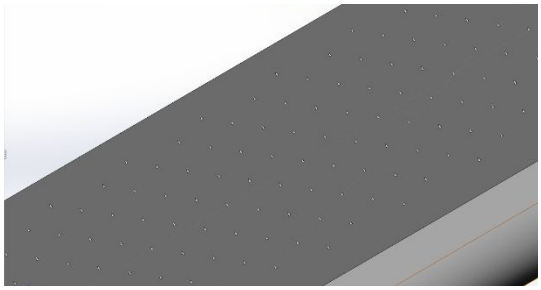
Team had a meeting with Dr. Nygren

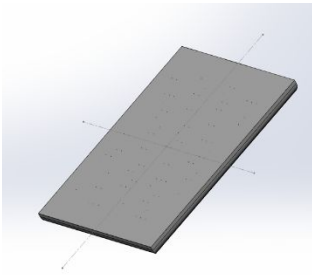
- Updates
    - 3D printing
        - Patch seemed to be a reasonable size according to Dr. Nygren
    - Diffusion assays
        - There were bacteria leakage that happened
        - Back up plans, solving issues
    - Meeting with Dr. UT
        - Thermoforming
    - Prototyping
        - Maybe we could use wax paper or parchment paper. See what happens.
        - Machine shop may not be able to help us since they will be closing soon for renovations. They would require us to send the SolidWorks model asap. They can't do heat sealing for us, but they can make the mould.
        - For the mould: instead of making 18 patches at a time, make it simple by making 1 at a time. Easier to manufacture, cheaper
        - Adhesive: use of heat
- Manufacturing
    - Materials for moulding
        - Suggestions he could give us
        - Moulding design he could provide
            - One at a time
    - Contacts for manufacturing
        - Companies: nope
        - Professors: nope
- Modelling
    - Diffusion model Noshin prepared
        - MATLAB code
        - ODE45 will help us get numerical values instead of having a function as a solution

        - C_2 degradation
        - Flux = moles/(m^2. s); production rate = moles/s
        - Start with assumed values for production rate and then move forward.
    - Diffusion model Nelly prepared
        - Will send the document to Dr. Nygren for checking

FRIDAY, AUGUST 5TH

- Mason completed a SolidWorks model of a previous microneedle prototype design
- Noshin and David continued to manufacture patches in the lab. Following Nelly's procedure, the made patches that were 1 cm x 1 cm





MONDAY, AUGUST 8TH

- Tiffany and Christine planned the week to perform the backing layer growth curves. These growth curves will be used to determine if cell growth is affected when gas exchange is limited
    - The Plan
        - Tuesday start the overnight cultures for the growth curves in the falcon tubes
        - Wednesday at 4:00 pm, 12:00 and Thursday at 8:00 am start inoculation for the tubes
        - Thursday start overnight cultures for the growth curves in the patch and perform growth curves for falcon tubes
        - Perform growth curves in the patch
    - Before starting the growth curves in the patch, soak the 3D printed models in 70% ethanol overnight

TUESDAY, AUGUST 9TH

- Tiffany started the overnight cultures for the growth curves in the falcon tubes
- Nelly and David made patch prototypes today. The procedures are as follows:

Materials:

- Strips of release liner (5cm x 13.2 cm)
- Strips of EVA membrane (5cm x 13.2 cm)
- Square cut outs of backing layer
- BIO PSA adhesive
- Cylindrical metal bar
- Masking tape
- Scissors, marker, ruler
- Flat plastic surface
- Iron
- Syringe + needle

Procedures:

A. Preparation of the adhesive layer

1. Have the materials ready. Perform the process under the fume hood as the adhesives contain heptane. Heptane is flammable and create vapor trails that may cause fire.

2. Note that the coated side of the liner is where the adhesive will be applied. In case you cannot figure which one is the right side, grab a marker and try to write on both sides of the liner. If the ink stayed permanently on the liner, you wrote on the uncoated side. The side where the ink just slipped through would be the coated side. It would be recommended to write which side is which to avoid confusion.
3. Draw a horizontal line on one end of the release liners (1cm from the end) with a marker. This end will be taped to keep the liner in place when the adhesive is being applied.
4. Draw three 3cm x 3cm squares on the release liners. Make sure to leave ample amount of space between the squares. Draw 1cm x 1cm squares inside the initial squares.
5. Take all the materials under the fume hood. Tape the end release liner on the flat plastic surface.
6. Apply the adhesive on the line initially drawn. Apply a constant pea-size amount on the line.
7. Using the metal bar, spread the adhesive onto the liner. Spreading using a metal bar will form a thin film of adhesive on the liner.
8. Wait for about a minute before placing the EVA on the layer.
9. Carefully place the EVA membrane strip on the adhesive layer.
10. Lightly tap the membrane to stick.
11. Wait for the adhesive to completely dry (1 hr - 3 hrs).
12. Cut out the squares.

B. Heat sealing the patch

1. Set the iron to the heat sealing temperature that was previously determined.
2. Place the backing layer on the prepared adhesive-membrane layer.
3. Carefully iron the sides of the layer. Avoid ironing parts of the 1cm x 1cm square centre drawn. This is where the nutrient rich media and bacteria will be stored.

C. Adding the media in

1. Obtain the required amount/volume of media to be stored in the patch using a syringe.
2. Carefully inject the media into the middle compartments of the prepared patches by poking a hole on one of the corners of the drawn center squares.
3. Heat seal the holes created by the needles.

***Check the whole document under https://docs.google.com/document/d/18uXLYuw3K3-0EAZaxPTyErA_iBWKPDveNloJplzlsIU/edit

WEDNESDAY, AUGUST 10TH

- The team went to the Foothills machine shop yesterday at around 2pm to consult with Peter Byrne. He was the person Dr. Nygren had referred during our last meeting. We had set the mould specifications for him to follow. He had also given us suggestions to further improve our mould. The mould will be ready for about a week. ($50 per hour of labour by the way ladies and gentlemen).
    - The mould will be made with aluminum and brass that may stick to our layers.He then recommended us to get a Teflon coating spray or something the same.
- Nelly finished adding her Powerpoint slides for the presentation on Monday. She also picked up a Dupont Non-stick Lubricant (with Teflon) from Canadian Tire.
- David and Nelly made more adhesive layers before leaving the lab. Some had thicker adhesives applied, some had thinner layers. They will see how this quality would affect adhesion or if it has any effect at all.
- Nelly tried one of the adhesive layer on her wrist. It took her a while to completely stick the layer on because it had thin adhesive coating. It also required applied pressure for it stick better. She had the layer on for 4 hours.
    - Result: The layer was somehow painful to remove because some of her hair were stuck on the adhesive. She said the pain felt like a degree lower than a wax sheet slowly being peeled off your skin. There was no redness or irritation that happened on her skin, but itchiness was felt when the layer was on.
    - Recommendations: Disinfect the skin area before patch application.

- Christine continued working on the animation for Maya today and edited our presentation for August 15th.
- Tiffany conducted assays in the laboratory today
- Tiffany had also tested in the lab if the 3D models would hold the initially calculated volume of media.
    - Results: The pockets exactly held 0.5 mL of liquid, while the content area held exactly 10 mL. Therefore, the 3D model is a good representation of our patch system in terms of holding capacity.

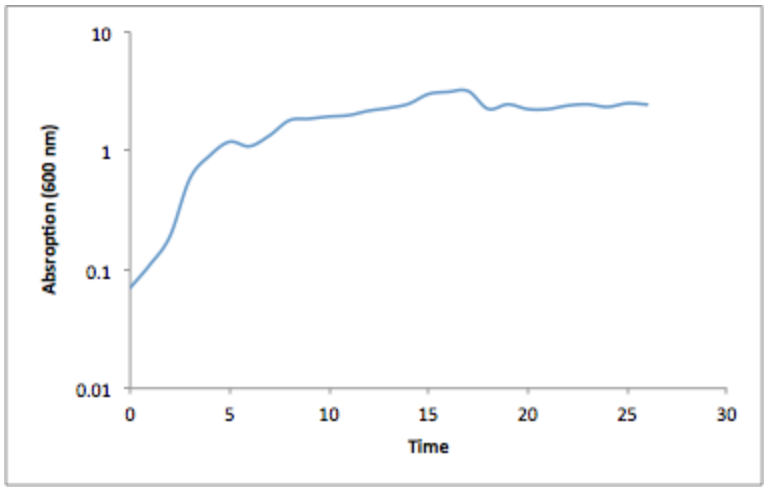
Thursday, August 11th

- Tiffany's focus today was to conduct the growth curve assays every 40 minutes.
- Meeting with Dr, Nygren
    - Potential questions here → Questions for Dr. Nygren document
- Meeting minutes:
    - Update:
        - Showed Dr. Nygren the full system prototype Nelly and David made
        - Continuing assays by Tiffany and Christine
        - Christine is working on her video
        - Noshin is working on her math model
        - Went to machine shop to get the thermoforming mold done
    - Modelling:
        - Dr. Nygren said Noshin is on the right track. He will email her to clarify some details.
    - Prototyping:
        - Dr. Nygren suggested talking to Dr. Jenne about the prototype and alter it to better suit the mice.
        - Ask Dr. Mayi to buy the heat gun on our own
    - Assays:
        - Try to make a connection between the assays and math models. Answer the question of why we do the assay, why we have the models, what the connections are. Cohesion is key.

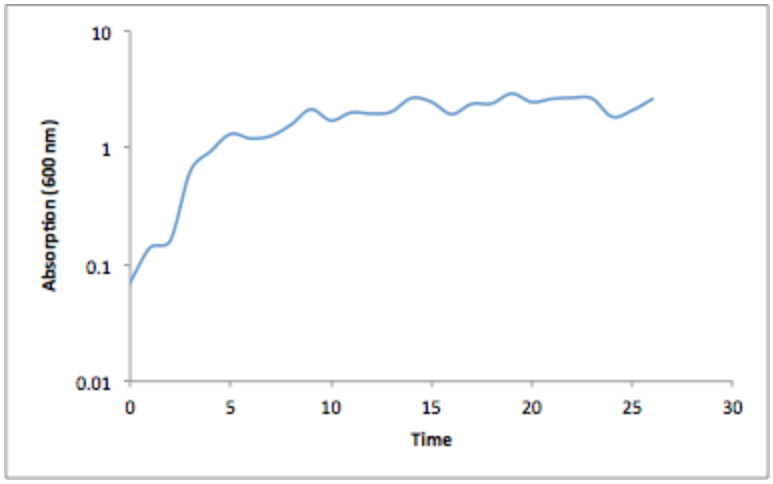
Friday, August 12th

- Christine and Tiffany worked on entering data values of their growth curve assays in a spreadsheet. They created time versus absorption plots.
    - Results:

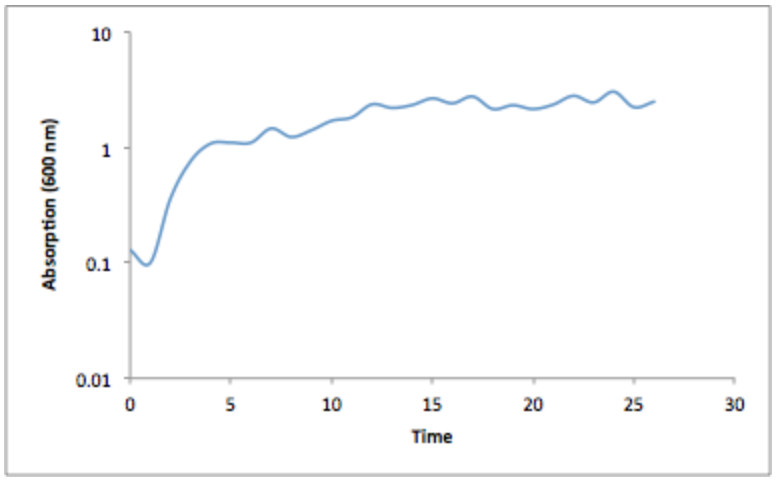
Tube 1



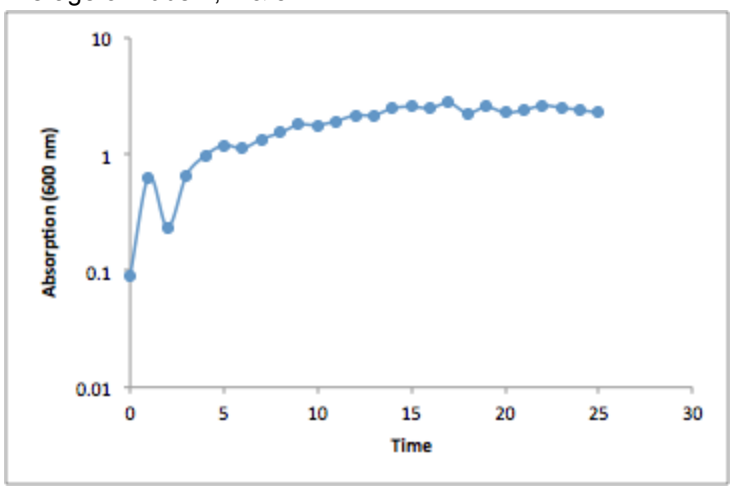
Tube 2



Tube 3



Average of Tube 1, 2 & 3



MONDAY, AUGUST 15TH

- Presentation day! What we basically did for today was preparing for our presentation and did run throughs.

- After the presentation: dead. There were challenging questions thrown at us. This means we still have things to be solidified before we can say we're done.
- We also asked our mentors for comments with regards to our presentation. They are as follows:
    - Instead of having a slide with all equations on them, why not just use words that tells our audience what the terms represent.
    - Maya animation. Changes to be made could be:
        - Making the adhesive liner take up the whole bottom area of the patch.
        - We're not using the indicator system to signal low media, but tells if the bacteria is activated.
        - After we have shown how the patch works, we can also extend the story by, say, an astronaut peels off the liner and apply patch, then wears this for this how many hours, and then through our modelling results we'll figure how long before we pop a packet and then what would happen next and, you know, the story goes on yadi yadi yada.
    - Our graphs must be able to speak for themselves, meaning that by the time we look at them, we know right away what it is trying to tell us.
    - Explain all the terms that we are talking about.
    - CONNECT ALL THINGS TOGETHER.
    - Use constants that are from the other teams. The values we get from our model runs must correlate to the models and numbers Chassis and Biotarget get. Both Rai and Dr. Nygren brought this up.
    - Our model should also have a flow.

Friday, August 19th

- David and Nelly made more adhesive laminates, preparing for more prototypes

Tuesday, August 23rd

- Tiffany continued doing her diffusion assays. The dialysis membranes came today as well and treated for testing. There were two types of tubing membranes that were used: 20 kD and 628 kD pore size membranes. However, these membranes are not heat sealable which would be a problem in manufacturing. There were other limitations that were mentioned and these can be found in the email Nilesh sent/cc'd us in. Tiffany will be performing diffusion assays for these membranes tomorrow.
    - Must be soaked in room temperature distilled water for 30 minutes instead of being boiled
    - "Just soak them- once you have figured out how to glue them together" -- Dr. Volgo
- Nelly made around 80 adhesive laminates.

Wednesday, August 24th

- Tiffany performed her diffusion assays experiment using the dialysis tubing membranes.
- Noshin worked on modelling and sent the codes to Dr. Nygren for checking.
    - She also asked Dr. Nygren if he could give us a contact who could help us with cost analysis for our patch.

Friday, August 26th

- Tiffany and Christine picked up the moulds from the Foothills machine shop.
- The results from dialysis membrane diffusion assays came out. There is still diffusion of bacteria through the membrane.

- The team also met with a PhD scholar, Xiaoan Li, whose research project focuses on water filtration using nanoporous membranes. Robert, a student who works in Li's lab, is also in the meeting.

Changing points in our Project

Monday, May 30th

Meeting with Dr. Dalton:

- Contact 3M for hollow microneedles, look at patents
- If he can find his microneedles he will give it to us
- Would have to consider shear stress, what if microneedles break off and go into the body
- Utah array - solid microneedle
- Lab on a chip - chips and tips
- Perhaps make microneedle system separate with valve
- Issues:
    - In microfluidic systems are bubbles - bubble will block channels
    - LB chamber, what's stopping the fluid from flowing directly out the microneedles? Perhaps use a mechanical system to control pressure, could also suck the media back in
    - Diffusion would be way too slow or not even occur, would need an external syringe pump
    - Leaving microneedles in for too long will cause immune response from body, wound infections
    - Need to determine how much pressure the membrane can take
    - Consider stress on microneedles, fact that skin is very mobile
    - Skin elasticity, skin varies by thickness based on ethnicity and age, also have to consider the hairs on the skin

Wednesday, June 22nd

- Meeting with Dr. Amir Nezhad
    - About Dr. Amir Nezhad's Research
        - Dr. Sanati-Nezhad' primary research interest involves BioMEMS, Microfluidics, Tissue Engineering, Micro and Nano Technology, and Lab-on-Chip.
        - His research group has focus on development of integrated bioinspired microdevices using microfluidics and tissue engineering approaches for disease modeling, biological systems modeling, and drug discovery.
        - Another research interest of his group is to develop point-of-care devices for testing infectious diseases, and portable tools for detection of plant and food pathogens.
    - On meeting with him, we were invited to his lab where he showed us the process of micro fabricating the labs on the chips and how he uses microfluidics in order to do so
    - The fabrication process is difficult. It requires a semi permeable membrane in the middle where the individual cells can be housed.
    - Surrounding the permeable membrane is silicone mold to provide structure and handling.
    - Although the chip looks simple, actual analysis requires a system of micropumps to transfer media, waste and byproducts across the system. This in turn is connected to a computer or microscope system for analysis.
    - In order to work for cells for 30 days, the first 6 - 15 days is incubating the cells and ensuring they are in a happy media. After that, you can start researching on the cells
    - He mentioned the importance of finding the optimal flow rate in the chip because anything above or below they flow, will disrupt the cells and kill them
    - In concerns with our project
        - He thinks the general idea of a patch system releasing various peptides into the body is very interesting and sees potential for collaboration with his lab
        - When asked about companies to order from he suggested Dow Corning
        - He stressed the importance of drafting up a prototype of our design in AutoCAD so he could better understand what our design will look like (not really? We asked him what he used for visual modelling and he said AutoCAD. However, he did stress

making a powerpoint presentation to show people we consult to give them a better idea of what we are doing)
    - Gave us access from his lab to purchase AutoCAD
- When asked about fabrication, he volunteered his services or that of the mechanical shop to create a prototype

THURSDAY, JUNE 23RD
Meeting with Dan and Dr. Mayi

- Contacted Porex for EVA samples - waiting for response
- Also discussed with Dan about assays we can perform for both the semi permeable membrane and backing layer
    - For the semi permeable membrane
        - Using a 15 mL falcon tube, we can fill it with overnight cultures and wrap the semi permeable membrane underneath the lid. We then invert it in a larger 50 mL falcon tube with water, salts and at the same pH as the blood and leave it to sit. After hourly intervals, we would take samples of the water and take the OD readings.
        - Another assay that we can do is to use a brute force method and apply the same setup as the first assay. However we would centrifuge it down and see at the extremes how well our semi permeable membrane works.
    - For the backing layer
        - Dr. Mayi suggested creating a small patch with overnight cultures and leave it to sit in conditions similar to those found in the ISS. We take an OD reading initially and then let it sit for eight hours before taking another reading at the end and comparing the growth of our cells. We can also measure the amount of moisture vapour by measuring before and after the mass of the patch.

        - Dan also suggested something similar to measure how well our cells grow. Using the small plates, we would aliquot some of our overnight cultures into two plates. We would then cover one with parafilm and the other with our backing layer. We then leave them to shake in the incubator. We take the initial OD and the final OD reading then of both to see how well our cells have grown

THURSDAY, JULY 21TH

- David replied to 3M, they sent me back an email with useful info. I forwarded to everyone:
    - Temperature and pressure depends on system:
        - 250F and 40 psi for their small single well sealer - 1 second so the liner doesn't warp.
        - However, multiple well sealer needs 300F, 50 psi
        - => May need a few trials to decide the parameters
    - They can create a crude prototype to give us some idea of usability
    - If making it ourselves, they recommend a sequence of steps:
        - coat adhesive onto the liner, laminate the membrane to the liner through a low pressure nip roller and lastly heat seal the backing to the lamination (on the membrane side) by placing the materials to be sealed onto a well type receiving fixture with silicone sealing gaskets and using a platen type seal plate above.
    - Our membranes are the most permeable. The main difference is the thickness between the 2. The thicker one (9716) will have a slower transmission rate. If nothing works, they'll help us pick out another one.

- Meeting with Dr. UT Sundararaj about manufacturing:

- He went over the project with us and said gel media might be safer when being punctured but diffusion might be compromised. However, he said at equilibrium everything should be the same.
- He approved our materials for the patch. He told us to consider the materials' solubility parameters to make sure they are compatible and nothing will dissolve into each other when heated. EVA and polyethylene are fine. We might want to check hexane/heptane in the silicone adhesive and EVA though, but it should be fine.
  - http://cool.conservation-us.org/byauth/burke/solpar/solpar2.html If delta is >+-2 it should be okay.
- He recommended using thermoforming for our mouse patches. The general idea is to make a wood "mold", heat our backing layer up, put it on, vacuum so it forms a reservoir, pour the media in, put the membrane on top, and then heat seal everything together. A flat iron for hair or something might work. It's also a good idea to apply heat on both sides.
  - Thus, we need to get in touch with the machine shop ASAP http://www.plasticsmag.com/thermoforming.asp?fIssue=Mar/Apr-03 some reference we pulled out from google today about thermoforming.

FRIDAY, JULY 22ND

- Dow Corning (finally!) responded to Nelly about the preparation of adhesives.
  - Basically, the actual adhesives are dissolved in heptane. This is the reason why handling it without precaution might cause irritation. The adhesive in its pure form is safe to use.
  - Easy enough, we just have to let the heptane evaporate so we can use the adhesives. The evaporation rate will depend on the temperature of the environment. In some patent documents she found online, companies dried the same type of adhesive in 100^{o} C oven for 2-3 minutes.

  - Note, we must do any adhesive assays under the fume hood.

WEDNESDAY, AUGUST 17TH

- Meeting with Dr. Jenne (August 17, 2016)
  - Mouse orders done on Wed the 24th potentially
  - Might take some time to do training, he's concerned about the timeline since he technically can't order mice until he got ethics done
  - He thinks that the biggest thing for us is mass spectrometry
  - Problems we might encounter:
    - Peptide not being found in the blood
    - If it's found in the blood, what if it's absorbed by other cells or organs
  - The most important thing is what we get out of it, what the results tell us, and our future solutions for it. It's okay if it fails.

  - We have to change the dimensions of our patch to make it narrower and longer.

MONDAY, AUGUST 22ND

- Modelling meeting with Dan
  - In our modelling story, this experiment would be in the beginning of the story. This would be use which peptide we are going to use. Once our peptide has gone into the bloodstream, this model would help us determine how fast our peptides would diffuse through the cells to do its functions.
  - Different forms of our peptide
    - How it would go through our membrane
    - Looking at the energy levels
      - Applying force to membrane through a bilayer

                - Very hydrophobic = low energy in the middle
                - Calculate energy
        - Size of the system
            - Peptides: we want to model the different forms of our peptide and see which diffuse the fastest
            - 9-residue monomer - linear (reduced form) and cyclic (oxidized form, ring, forming a disulfide bond = might affect diffusion, should it be reduced or not)
            - TD1 tag: compare that to BBI (combination of the 2 may have a better effect)
            - KSCI + monomer + F at the end
        - Having a negative control
            - Like a sodium ion (+ve)
            - Hormone that readily diffuses through
        - Another important part: bilayer
            - Simulate the actual membrane
                - One type of human bilayer = have a smoother curve
                - Or a hydrocarbon bilayer/disc
                - Talk to them if we could have a simple bilayer with true phospholipid
                - Explicit water on the outside
                - Size of the environment (10nm cube, etc.)
        - Ask if there is data already existing

FRIDAY, AUGUST 26TH

- The team also met with a PhD scholar, Xiaoan Li, whose research project focuses on water filtration using nanoporous membranes. Robert, a student who works in Li's lab, is also in the meeting. He was a previous member of the winning iGEM team in 201_. Here's what we talked about:
    - Ask Dr. Mayi or Deirdre about other membranes that we could test
        - Examples
            - centricon: small scale version, 5kD (ask Goodarzi's godmother): not as big, small scale purification, good for centrifuge works
            - filter sterilization membranes (0.22 micron)
    - Watch 2011 NASA - video
    - figure out what carbon nanotubes we need, they got some. They have PPL, carbon nanotubes. Once we've tried everything and still fail, then we should ask them.
    - Other ideas
        - dissolving O_2 on the actual membrane
        - We need less than 200 nm of pore sizes
        - SEM, AFM: contact Matthias Amrein - does lung cells; Michael (runs SEM)
            - use something conductive, Teflon is not conductive
            - thin sheet of gold
        - figure out what materials we would need at the moment

PROVIDERS

COMPANIES INVOLVED:

3M

- Provided us with all the sample materials used in the team's assays
- The following materials are as follows:
    - 3M CoTran™ 9722 Backing Polyethylene Monolayer Film
    - 3M CoTran™ 9719 Backing Polyethylene Monolayer Film
    - 3M CoTran™ 9716 Ethylene Vinyl Acetate Membrane

- 3M CoTran™ 9728 Ethylene Vinyl Acetate Membrane
- 3M Scotchpak™ 1022 Release Liner Fluoropolymer Coated Polyester Film

Dow Corning

- Provides us with the adhesive samples used in our patches.
- The following materials are as follows:
  - Dow Corning BIO-PSA Silicone-based Adhesive 7-4101
  - Dow Corning BIO-PSA Silicone-based Adhesive 7-4201
  - Dow Corning BIO-PSA Silicone-based Adhesive 7-4301

Collaborations

Thursday, July 7th

- The team continued working on the diffusion model by either researching the equations further to see if there is another perspective we can take on the model or writing a code that can be used in MATLAB
- Nelly was able to get a graph of the result of her MATLAB code [Insert image or comments here about the result Nelly!]
- Meeting with Waterloo
  - The modelling team met with the UWaterloo Modelling leads to discuss a possibility for collaboration
  - They are working on a project that uses yeast to take advantage of the higher readthrough rates during prion response
  - There is not very much commonality between the two projects but one potential would be a collaboration on their protein aggregation model as it would involve transcription rates
  - However after discussing, it may not be worth the collaboration between us and Waterloo

iGEM Collaboration UCalgary & UWaterloo MM
07 JULY 2016 / 4:00 PM

ATTENDEES

- Zoë Humphries, Emily Watson, Tiffany Dang, Nelly Mendoza, David Nguyen, Sid Goutam and Nilesh Sharma

BRIEF INTRO

UWaterloo Project Summary

- Creating a system that takes advantage of higher readthrough rates during [PSI+] state of prion response
- Model of iGEM collaboration network from 2015
- Model of plasmid retention - metabolic load of expressing our fusion protein
- Model of protein aggregation - how the prions are distributed through generations

UCalgary Project Summary

- Working a transdermal delivery system to deliver peptides to the body
- Specifically working on the delivery of the Bowman Birk Inhibitor which as shown radioprotective effects
- Modelling the diffusion of BBI into the body to determine the initial concentration needed to be produced

- Model of the required transcription rates to produce the required amount of BBI or what a reasonable amount of BBI can be expected

MORE DETAIL ABOUT WATERLOO MODEL

- Gene Retention Model
- Determine number of copies based on fluorescent protein in plasmids
- Retention is dependent on metabolic load, stability of plasmid,
- Quantify using fluorimetry
- Protein Aggregation Model
- Model how long it takes for aggregation to form (help lab subteam)
- No differential equations as of yet
- Probabilistics important for chance of inheritance in daughter cells
- Quantify aggregation via Western blots
- System should break down prions, will model this disassembly
- Quantify breakdown via GFP (sup35 prion protein begins functioning again)

MORE DETAIL ABOUT CALGARY MODEL

- Diffusion Model
- Model how long it takes for the diffusion of BBI to reach steady state
- Compartmentalized the diffusion system into three components: the patch to the skin to the blood stream
- No differentials equations at this point but trying to use Fick's Law in MATLAB to see if we can analytically solve for the steady state
- Also using this model to determine the initial concentration needed in the patch for the desired amount of BBI to be found in the blood stream
- Transcription Rate Model
- Model to determine how fast the peptides are being produced inside the patch and how varying transcription rates will create the necessary concentration of peptide needed in the patch

ACTUAL ITEMS FOR COLLABORATION

UCalgary Assistance

- Help starting with the transcription rate models to determine how fast the peptides are being produced and what is a reasonable initial concentration based on these rates
- Mentoring for the diffusion model. This would be in the form of bouncing questions with Waterloo to see if we are on the right track and if there are any considerations we need to take
- With the gene retention model, UCalgary can help by collaborating on the transcription rates specifically

UWaterloo Assistance

- Gene retention subgroup most likely to benefit from assistance
- Optimising expression of plasmid number over gene copy number in plasmid
- Modelling expression of gene, how quickly the plasmid is lost during normal and prion states ([PSI+] condition)
- Dr. Brian Ingalls works on gene retention in bacteria, is a great resource
- Transcription rates would benefit gene retention and protein aggregation

ACTION ITEMS

- Use Google Drive to maintain collaboration docs
- UWaterloo will send update e-mail to math subteam & CC UCalgary

MANUFACTURING

WEDNESDAY, AUGUST 24TH

- Dave and Nel worked on manufacturing today. David had figure out a way to properly make our mice testing patches. The protocol is as follows:

  Procedure:

  1. Prepare the adhesive laminates following Nelly's procedure
  2. Plug the iron in to preheat.
  3. Look through the adhesive laminate under a light source and pick out and most even area. Sketch the 1 x 1 cm square on the liner at the center of the area
  4. Trim the laminate to roughly 1.5 x 1.5 cm
  5. Lay the laminate membrane-side up on the iron board, and the backing layer on top
  6. Iron 1 side of the patch up to the sketch. To make the seal even and bubble free, when sealing, apply more pressure on the side of the iron that's touching the line, then tilt the iron so that side of the iron lifts up slightly, then press it down again, after each time, move the iron away from the line to the edge of the patch. The seal should look clearer than the center. If not, then the iron is not hot enough, redo using a different side of the iron until it's clear
  7. Seal the other 3 edges. Now the patch should be stuck to the iron board, leave it there. Repeat the process for another 2 patches.
  8. Remove the first patch from the iron board. Prevent peeling the patch off the liner by sliding a finger under the patch after you peel off 1 corner when you reach the liner.
  9. Hold the patch diagonally, vertically, backing-side facing you. Bend it slightly so the backing folds towards you. The backing at the center (not sealed) should bend and separate from the membrane, creating a thin pocket running diagonally from the top corner to the bottom corner.
  10. Fill the syringe with water, use the needle to poke a hole at the top corner of the patch, make sure the needle did not poke anywhere else and the needle is in deeper than half of the patch.
  11. Inject the water slowly, keep a bend in the patch so we have a pocket, fill it from the bottom corner, tilt the needle and the patch to get the other 2 corners, then fill it nearly to the top corner. A good volume should be 0.06 - 0.08 mL
  12. Remove the needle straight out, a drop of water may leak out, dry it lightly with paper towel, don't push on it
  13. Lay it on the iron board. Make sure there's no water droplet visible. If there is, dry it gently with a paper towel, then iron on the hole for about 1-2 seconds
  14. Press on the patch slightly to see if there's still any leaks, if yes, dry the droplet of water with paper towel and re-iron the corner until there's no more leaks.
  15. Now the patch is done, trim the sealed sides with scissors to approximately half of the original side dimensions. Round off the sharp corners
  16. Cut a bandage in half, horizontally, then cut it vertically to create 2 small rectangles, with the sides approximately to be twice of the sealed sides of the patch
  17. To put the patch on a finger, remove the liner, put it on and press all edges lightly but firmly until all edges stay down
  18. To reinforce the patch, put the 2 bandage pieces on both sides that go down to the sides of the finger

***Note:** A patch with "YES" written on it is on the iron board. It should show you the size of the patch, how clear the seals get, and how I seal the hole. You can ask Tiffany about the process of applying since she had 5 on her fingers today.

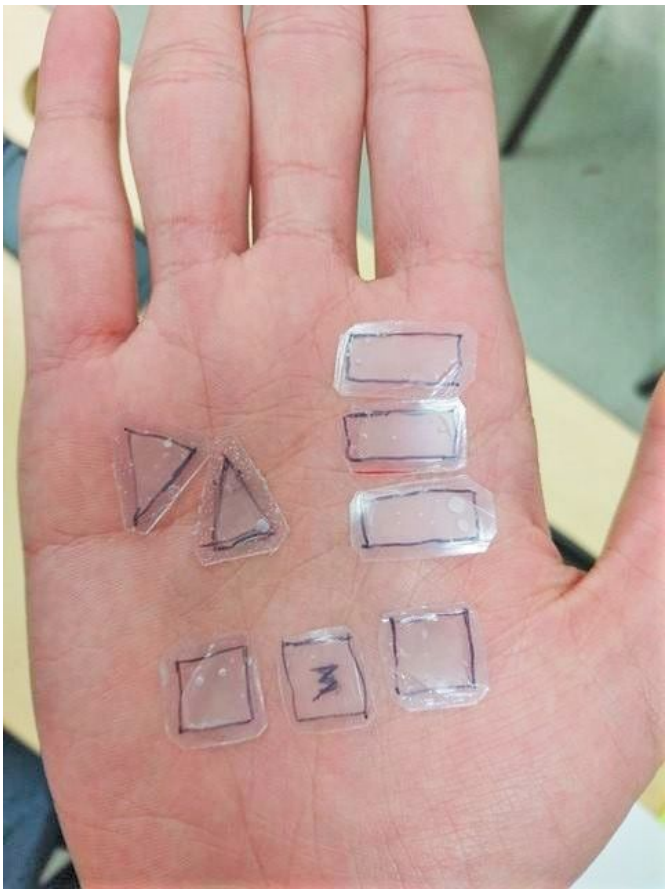
***Note 2:** This is a pretty reliable method. The seal works great most of the time. Poking more than 1 hole in the patch happens once in awhile. The patch stays on well most of the time. The water will leak when you put on if the seal is not good enough, or if there's another hole you accidentally poked.

Wednesday, August 31st

- David made some more mice patches for Thursday

Thursday, September 1st

- David put 3 patches of different shapes (square, rectangle, and triangle) on the mice in Dr. Jenne's lab and reinforced them with tissue glue.

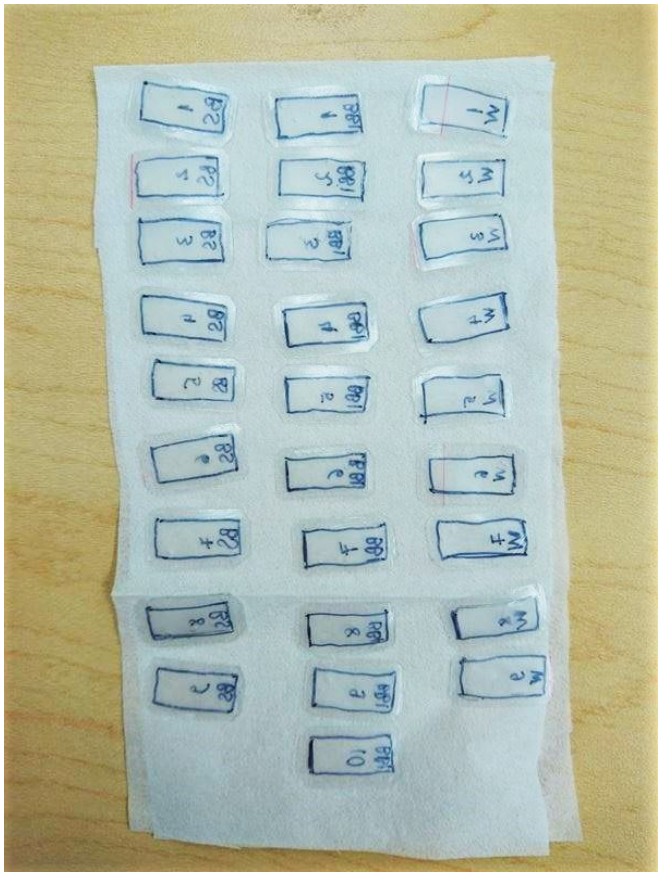


Friday, September 2nd

- All 3 patches from yesterday survived. The rectangle and triangle worked better than the square. Rachelle from Dr. Jenne's lab said the rectangle looked best.

Tuesday, September 5th

- David created 28 patches for the mouse testing with 9 containing water, 9 containing bacteria and 10 containing pure BBI



RESEARCH

Papers:

http://www.ncbi.nlm.nih.gov/pubmed/21139238

- Hydrophilic large molecular compounds
- Via hollow microneedles
    - Loaded into lower epidermis and superficial dermis
- Release rates (Fick's Law of Diffusion)
    - Increase volume of FD-4 injected - faster the FD-4 release rate from skin
    - Release rate increases when FD-4 given in multiple injections
    - Large molecule = lower release rate from skin
- Silicon - useful material?
- Questions:
    - So is the drug being released secreted out of the skin after secretion?

http://www.ncbi.nlm.nih.gov/pubmed/22575858

- Sections 2.4, 3.1,2
- Pressure gradient - greater pressure causes drug to flow through
- Diffusion gradient
- Questions:
    - Based on methods of delivery - silicon; have to test and make sure peptide/fluid/bacteria doesn't react with silicon

    - Simulate a large scale prototype of design to test if we can use pressure/diffusion to get the peptide to flow through
- Problem:
    - For microneedles that use multiple needles (our suggested form):
        - 1 microneedle leaks
        - Pressure can't be equally applied to all needles
        - Fluid won't flow through all microneedles equally
    - The small size of microneedles - drugs given on/within microneedles limited to microgram dose
- Applications of microneedles
    - Biotherapeutics, drugs (peptides, proteins, DNA,RNA)
- Hollow microneedles - used for insulin delivery in both rats and humans
- Diffusion and active infusion worked, decreased blood-glucose levels after injection
- Humans found it to be:
    - Decreased pain
    - More preferred (compared to normal needles)
    - Increased insulin pharmacokinetics almost 2-fold - may cause better control over post plasma glucose levels
    - Had faster insulin absorption and enabled more rapid onset and offset metabolic effect on blood glucose levels than injection

https://www.researchgate.net/publication/228562173_Side-Opening_Hollow_Microneedles_for_Transdermal_Drug_Delivery

- Proposed design - measurements for diameter length of needle, etc.
- pump/syringe for injection
    - Need to test diffusion; if diffusion doesn't work, use hand to press
- Overdose problem
    - Reservoir patches give tighter control of delivery rates but can have an initial burst of drug release. If the membrane is damaged, there is also a risk of sudden release of drug into the skin and overdose as potentially a larger area of skin is exposed for drug absorption.
    - In a matrix patch, the active ingredient is distributed evenly throughout the patch. One-half of a patch will have half the original surface area and deliver half the original dose per hour. The matrix patch carries less risk of accidental overdose and offers less potential for abuse than the reservoir system.

http://ieeexplore.ieee.org/stamp/stamp.jsp?tp=&arnumber=659727

- The principle of operation is based on the pressure barrier that develops when cross section of capillaries change abruptly in neck and expansion regions. This type of gating device in its normal state can only stop flow, but it can be electrically triggered to re-establish flow when used in combination with two electrodes.
- Mechanics: The capillary stop consists of a region of the tube that is necked down followed by a sharp enlargement. When the liquid as first introduce in the reservoir, it wicks in the necked region and abruptly stops at the neck of the outer edge preventing any outer flow. The pressure barrier provided by the stop can be overcome by external pressure to re-establish a flow.

MATERIAL RESEARCH

TUESDAY, MAY 31ST

http://images.alfresco.advanstar.com/alfresco_images/pharma/2014/08/22/ba2e9668-a586-4daf-9179-70f220be6e56/article-18600.pdf

PSA = Pressure Sensitive Adhesives

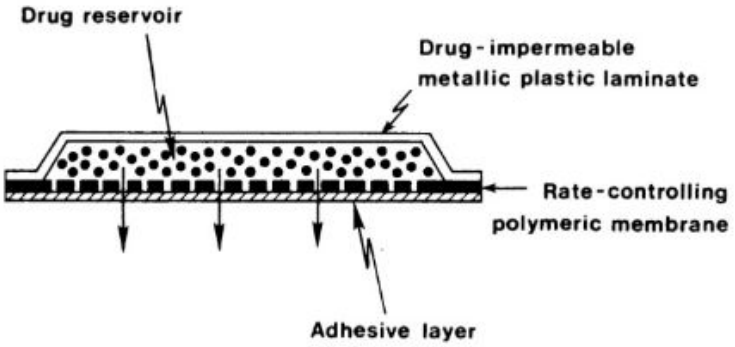
- Materials that adhere to skin with application of light pressure and do not leave residue upon removal
- See paper for materials used
    - Acrylic-, polyisobutylene- & silicone based adhesives commonly used in transdermal patches
- Increasing the polymer content provides a softer and tackier adhesive, whereas higher resin levels result in lower tack but higher adhesion and resistance to cold flow.
- Release liner is peeled off = also has the adhesive properties

Idea to prevent water from leaking out:

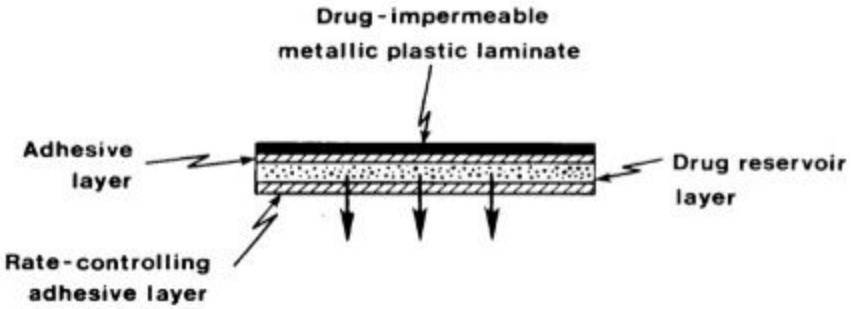
- Adding some sort of liquid in pores that will let peptide through and not let media mix with it
- Gel-like fluid for media

Design of patches:

- Polymer membrane partition-controlled TDD systems
    - There is a constant release as long as concentration is maintained, but release rapidly decline when device approaches exhaustion



- Reservoir system
    - Same concept
- Drug in adhesives
    - I don't think this is applicable for us, but would put it here just for reference



- Micro reservoir type
    - Suspension of drug with aqueous solution of water-soluble liquid soluble polymer
    - Homogenous dispersion of drug suspension in a lipophilic polymer (silicone elastomer)
    - Example: nitroglycerin patches

Competitors:

- TEPI Patch
    - Articles:
        - http://futurerxdream.com.ng/ibuprofen-patch-heralds-side-effect-free-drug-future/

        - http://www.painnewsnetwork.org/stories/2015/12/9/new-skin-patch-delivers-pain-relief-with-ibuprofen
    - 24 hour medication
    - Constant drug delivery for 24 hours
    - The drug is dissolved into the adhesive layer which helped it to release the drug in a steady rate and to take up more drug
    - Will be used for pain medication, so this will be applied to the **SPECIFIC** area where pain is felt
    - Will be out in the market for 3 years

Wednesday, June 1st

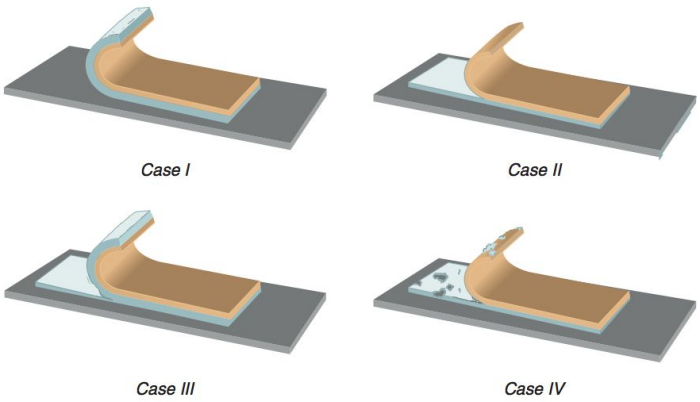
Adhesive layer research:

- https://geckskin.umass.edu/
    - Super-adhesive based on mechanics of gecko feet
    - Leaves no residue
    - Not sure if we can get our hands on this though…

Friday, June 3rd

http://www.tandfonline.com/doi/pdf/10.1517/17425247.2012.637107

- PSA's fall into three categories: solvent based, water based and hot-melt
    - Solvent based are traditionally used in patch production
- o Water based and hot-melt are more beneficial for skin irritation, sensitization and environmental contamination risks
- o Polyisobutylenes are better for allergenicity compared to acrylics and silicone-based
- Patch failures:
- o Case I: Adhesive failure
- o Case II: PSA doesn't adhere to backing layer
    - o Case III: matrix has good adhesive strength, poor cohesive strength
    - o Case IV: adhesive and cohesive failure



- PIB-based adhesives:
    - o Disadvantage: easy oxidation and low air and water vapour permeability
- Acrylic-based adhesives:
    - o Colorless and transparent
    - o More resistant to oxidation
- Silicon-based adhesives
- When testing in vivo performance:
    - o Need to find an artificial material that is able to simulate continuous variations of skin humidity - related to critical surface tension, surface roughness and deformability

- Skin deformability is most critical to consider
- Effects of relative adherend humidity on peel adhesion performances can be studied using collagen-coated plates
- When peeling off a patch, need to consider tensile deformation, bending stiffness and substrate deformation
- Stress distribution on skin deformation was measured in vivo by tension, torsion, suction and indentation tests

Some Market Research:
Paper: **Challenges and opportunities in dermal/transdermal delivery**
From <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2995530/>
PATCHES that is applicable in our subgroup:

- Clonidine patches
  - Catapres TTS® was introduced in **1984** for high blood pressure as the first 7-day patch system
  - "The patch should stay in place during showering, bathing, or swimming for **a full 7 days**." From <http://www.mayoclinic.org/drugs-supplements/clonidine-transdermal-route/proper-use/drg-20073656>
  - Clonidine is in a class of medications called centrally acting alpha-agonist hypotensive agents. It works by decreasing your heart rate and relaxing the blood vessels so that blood can flow more easily through the body. From <https://www.nlm.nih.gov/medlineplus/druginfo/meds/a608049.html#precautions>
  - How did they solve the problem with loss of adhesion? "If the clonidine patch loosens while wearing it, apply the adhesive cover that comes with the patch. The adhesive cover will help to keep the clonidine patch on until it is time for the patch to be replaced. If the clonidine patch significantly loosens or falls off, replace it with a new one in a different area. Replace the new patch on your next scheduled patch change day."
    From <https://www.nlm.nih.gov/medlineplus/druginfo/meds/a608049.html#precautions>

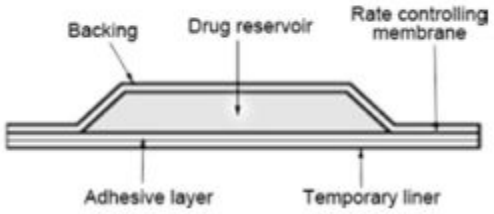
- Climara patches
  - Treating conditions due to menopause (eg, hot flashes; vaginal itching, burning, or dryness), treating vulvar and vaginal atrophy, and preventing osteoporosis. It is also used for estrogen replacement therapy after failure of the ovaries and to relieve symptoms of breast cancer. From <http://www.drugs.com/cdi/climara-weekly-patch.html>
  - Method of application: A new patch should be applied to your skin on the same day once a week (i.e., the patch should be changed once every 7 days)
    From <http://chealth.canoe.com/Drug/GetDrug/Climara>

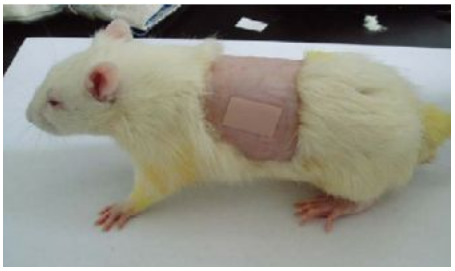
TUESDAY, JUNE 7TH
isosorbide dinitrate (ISDN) Patch
http://www.scielo.br/pdf/bjps/v51n2/1984-8250-bjps-51-02-00373.pdf
***They are dealing with a drug called isosorbide dinitrate (ISDN) which is used as vasodilator for angina, congestive heart failure, and esophageal spasms.

What they are trying to create:

- **Acrylate polymers** = they used this as the rate controlling membrane, as this polymer has not been extensively used in patches before
  - **Why use this**? keep drug release for at least 48 hours at constant rate

Here's how their patch looks like: poor mouse :'(





- Patch was stored in a sealed aluminium pouch = minimise the loss of solvent

Part of the Patch	**Brand or what material was used**	**Why was it chosen**	
Backing layer		Same material as what is existing	Temporary liner and the backing layer were then **heat-sealed** and cut to the appropriate sizes
Temporary Liner	**3M, Scotchpak 1022**	Packaging purposes, protects adhesive	Adhesive solution was coated onto the temporary liner (**3M, Scotchpak 1022**), allowed to dry completely
Rate controlling membrane	Polyacrylate membrane (made from scratch), synthesised by 2-hydroxy-3-phenoxypropylacrylate, 4-hydroxybutyl acrylate and diethyl maleate	To keep drug release for at least 48 hours at constant rate Better permeation, does not involve degradation, erosion or dissolution of the polymer	These are non-degradable polymers Thickness: 14 microns Pore sizes are fabricated randomly by polymer chains
Reservoir layer	75% PVA, 10% ISDN and 5% urea	Showed better permeation Permeation rate is increased by 25.4 fold, a 31.1-fold cumulative release after 24 h, and a 30.8-fold cumulative ratio of release at 24 h compared to EC	Also has the penetration enhancers
Adhesive layer	PSA (pressure sensitive adhesives): 5% ISDN dispersed in the mixture of PVP K90, PEG400 and gelatine	This whole combination helps enhance drug permeation	
THEIR CONCLUSION	This presented a longer release time at a sustained release rate Would promote patient satisfaction Sustained release rate, owing to the rate-controlling membrane, A higher loading-drug amount, owing to the separated drug reservoir layer Easier to tune release rate and release time to achieve the prediction		

Patch 2:

Contraceptive patch http://www.google.com/patents/WO2013112806A2?cl=en
Dosage: 20 mg per day, $14 for a full month of medication

Pros about this patch		**Cons about this patch**
Entire patch is flexible enough to effectively and comfortably adhere to contoured sites of the body Used destrogel mixed with carriers		
Part of the patch	**Material used**	**Why did they use it**
Backing layer		
Release liner		
Adhesives	Duro Tak® 87-4098 by Henkel Corporation., Bridgewater, N.J. Comprises a certain percentage of vinyl acetate co-monomer PIB adhesives such as 0.1 to 30 wt% PVP (i.e., povidone) or a PVP co-polymer such as PVP/VA (i.e., copovidone) as a humectant and plasticizer	PVPs are very hydrophilic as compared to PIBs, which are hydrophobic, has an ability to absorb moisture. The use of PVP copolymers, such as PVP/VA, can improve compatibility with other polymers and modulate the water absorption.

Recommendations:	**WHY**
use of water soluble polymers is generally **less preferred**	Would cause dissolution or erosion of the matrix Would affect the release rate of the desogestrel Would affect capability of the dosage unit to remain in place on the skin
Incorporate cross-linking monomeric units or sites	Would solve problems with polymers having **glass transition** temperatures below room temperature which are used to form a polymer matrix as the transdermal desogestrel-containing composition
In development of suitable polyisobutylene PSAs, one consideration is that PIBs are not crosslinked so they flow slightly.	Within a patch, that slight flow can cause an unsightly ring around the patch when it is worn for several days.

A higher content of high molecular weight PIB in the PSA formulation. **Polybutene in certain PIB formulations, such as the Oppanol B-12 functions as a plasticizer to allow for incorporation of more high molecular weight PIB. Mineral oil can be used as a plasticizer for the same purpose.	

Wednesday, June 8th

http://www.technicaljournalsonline.com/ijpsr/VOL%20II/IJPSR%20VOL%20II%20ISSUE%20I%20JANUARY%20MARCH%202011/IJPSR%20VOL%20II%20ISSUE%20I%20Article%2019.pdf

Polymer requirements:

- Biocompatible + Chemically compatible -> with both drug and body

Different companies use different polymer systems:

- Alza Corporation: EVA (Ethylene Vinyl Acetate) or microporous polypropylene
- Searle Pharmacia: Silicone rubber

  Backing Layer: Most common is Polyester-polyethylene composite

  Rate controlling membrane:
    - EVA: The percentage of VA can be manipulated. Higher VA -> higher permeability and higher polarity. Maximum VA is 60% by weight (or else glass transition temperature will increase)
    - Silicone rubber: Biocompatible, ease of fabrication, high permeability (especially steroids), free rotation around silicone rubber backbone -> Low microscopic viscosity within polymer

  Adhesive: (also referred to as PSA) We talked about PIB today

  Release liners: Fluoropolymers

Monday, June 13th

Media Release Material

Water Soluble Materials

- Aquasol uses a mixture of sodium carboxy cellulose and wooden pulp to create the water soluble material
- Aquasol specifically designs their packaging to biodegrade over time or with the introduction of water at any various temperature
- Monosol is another company which specializes in the use of water soluble packaging and dispersible materials. The water soluble film is made from PVOH (Poly Vinyl Alcohol)
- Like Aquasol, the material is water soluble at all temperatures. At higher temperatures, the material is more soluble. That is why they suggest moderate temperatures of 10-20 degrees celsius with relative humidity of 30-60%
- With the material being quickly degraded, water soluble materials are not the best materials to use for the media release. The material however does not harm the environment as the bacteria naturally found in wastewater can break it down into harmless components
- As well, there are no specifics about their material as you have to customize it to your needs

Polyethylene and Bubble Wrap

- An alternative would be to use a single layer of polyethylene used for bubble wrap. By filling the media bubble with media and air, it'll form a pouch that can be popped.
- Polycell makes a bubble wrap called Oxo-B Eco Bubble which incorporates their Reverte Oxo Biodegradable into their polyethylene resins. After discard, through the use of substantial UV light, oxygen and/or heat it will break down in smaller pieces. These smaller pieces are then broken down

further by the ingestion of bacteria and through respiration will degrade the plastic into carbon dioxide and water.
- Will look into plastics that degrade over time

Membrane:
- Contacted Dr. Mintchev for a meeting about transdermal patch + materials.
- Reading this paper: page 13

https://www.ualberta.ca/~csps/JPPS8(1)/N.Udupa/glibenclamide.htm
    - This paper tested EVA 2%, 9% and 19% both *in vitro* and *in vivo*. The trend held true that the higher the % of EVA, the more drug diffuses.
    - They also tested ethyl cellulose, Eudragit RS-100 and Eudragit RL-100 but only *in vitro.*
    - The drug was glibenclamide to treat diabetes.

    Reading this atm:
    http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2014/12/WC500179071.pdf

Release Liner:
- Needs to be chemically inert with drug penetration, penetration enhancer and water
- 3M Scotchpak 1022
- 3M Scotchpak 9741
- 3M Scotchpak 9742
- 3M Scotchpak 9744
- 3M Scotchpak 9755
    - Fluoropolymer Coated Polyester Film
    - Good for release with silicon skin contact adhesives, acrylate, PIB and rubber based PSA
    - Excellent chemical stability

Adhesives:
Links:
1. https://label.averydennison.co.za/content/dam/averydennison/lpm/na/en/doc/home/resource%20center/Adhesive%20Overview(1).pdf
2. https://www.researchgate.net/publication/51881833_Adhesive_properties_a_critical_issue_in_transdermal_patch_development_Expert_Opin_Drug_Deliv_933-45

The typical adhesive properties include:
- **Initial Tack** - The immediate holding power of the label upon contact with the substrate. A label with high initial tack will grab the substrate quickly. A label with low initial tack will exhibit a low level of adhesion when first applied and may remove cleanly.
- **Ultimate Adhesion** - The ultimate or maximum holding power that the label will achieve as the adhesive penetrates into the substrate. The time required to obtain ultimate adhesion may depend on the stiffness (shear) of the adhesive, the roughness of the substrate and the temperature of the environment.
- **Shear Resistance** - A measure of the internal cohesive strength of the adhesive. The shear of the adhesive is an indication of how soft an adhesive is. A low-shear adhesive (soft) has more of a tendency to flow (resulting in higher initial tack), and has a higher chance that the adhesive will split apart if put under stress. A high-shear adhesive (firm) is less likely to split under stress because of its good internal cohesive strength, and will be less likely to flow (possibly lower initial tack).

- Solvent-based
    - Traditionally used in patch production
    - Good for extended wear
    - Provide tighter hold
    - Stingy

- Silicone-based
    - High oxygen/gas permeability
    - Low pain upon removal to sensitive skin
    - Can be customized to improve chemical compatibility and stability with cationic drugs
    - Increased diffusivity
    - Tendency to cause drug crystallization
- DOW CORNING® BIO-PSA 7-4101 SILICONE ADHESIVE, DOW CORNING® BIO-PSA 7-4201 SILICONE ADHESIVE and DOW CORNING® BIO-PSA 7-4301 SILICONE ADHESIVE that way you can compare the different levels of tack. They are all amine compatible with heptane as the carrier solvent."

Dow Corning BIO-PSA Silicone Adhesives	Resin/Polymer Ratio	Silanol Content	Typical Solids Content %	Solvent
Standard BIO-PSA				
7-4401*	65/35	High	60	Heptane
7-4402*	65/35	High	60	Ethyl Acetate
7-4501	60/40	High	60	Heptane
7-4502	60/40	High	60	Ethyl Acetate
7-4601	55/45	High	60	Heptane
7-4602	55/45	High	60	Ethyl Acetate
SRS7-4501	60/40	Medium	70	Heptane
SRS7-4502	60/40	Medium	60	Ethyl Acetate
SRS7-4601	55/45	Medium	70	Heptane
SRS7-4602	55/45	Medium	60	Ethyl Acetate
Hot Melt 7-4560	60/40	High	100	None

Math Research

Diffusion:

Factors affecting diffusivity:

Search up: Chapter 2: Overview of Controlled Release Mechanisms by Ronald A. Siegel and Michael J. Rathbone

- Depends on **size of molecule** and **medium**, as well as the membrane that we're using
- For a hard spherical molecule diffusing through:
  Equation: D = kT/(6*pi*a*n)
  - a = molecule's radius
  - T = absolute temperature (K)
  - n = solvent viscosity
  - k = Boltzmann's constant (this accounts for intensity of thermal agitation)
- In terms of the medium: in *free volume theory*, each drug, solvent, and polymer molecule contains an impenetrable core that is surrounded by nanovoids, called free volume
  - Thermal motions cause the size of voids to fluctuate. Occasionally, a void becomes large enough for a diffusing molecule to move into or through it
  - Free volume of a matrix depends on the its composition
  - Free volume can also be increased substantially by sorption of small molecules, such as water. (sorption is the physical and chemical process by which one substance becomes attached to another)
  - For a molecule diffusing through a water-swollen hydrogel, diffusivity of drug is affected by the viscosity of the water space and also by obstructions placed in the drug molecule's path by the hydrogel chains

Monday, June 6th

What we need to model:

- Oxygen diffusion through the backing layer (outside to inside and vice versa)
- The amount of peptides that go through the rate membrane
- How much force would we apply to the pouches for them to break
- Mixing of media in patch and in pouches
- Start modelling the system
  - Play around with the sizes of the packets
  - Do math around, having some idea about how something works would be necessary, at least we have an idea how it works
  - Determine what we're actually trying to solve
  - MATLAB = useful way we could deal with our model - trying different values for a parameter we don't know about and see how it changed, identify what it represents, it's a framework of understanding which parameters are important

Friday, June 10th

Rate of release of therapeutic agents from reservoir transdermal systems:

- https://www.researchgate.net/file.PostFileLoader.html?id=55f196f8614325b8798b456f&assetKey=AS%3A272146475778050%401441896184273
- Assuming mechanism of drug delivery involves these steps:
  - Drug dissolution within the reservoir matrix
  - Drug diffusion and partitioning into the membrane
  - Drug diffusion within the membrane and partitioning into the adhesive layer
  - Drug diffusion within the adhesive and partitioning into the stratum corneum

- Rate controlling membrane controls drug diffusion into the adjacent adhesive layer and therefore is the rate-limiting step in the diffusion process

$$\frac{dM}{dt} = \frac{K_{m/r} K_{a/m} D_a D_m}{K_{m/r} D_m h_a + K_{a/m} D_a h_m} C_R$$

Where:

C_R is the drug concentration present in the matrix,

$K_{m/r}$ and $K_{a/m}$ are the reservoir/membrane and membrane/adhesive partition coefficients,

D_m and D_a are the diffusion coefficients of the drug in the rate controlling membrane and the adhesive layer, and

h_m and h_a are the thicknesses of the rate controlling membrane and the adhesive layer.

https://www.quora.com/Why-arent-patches-used-as-drug-delivery-systems-more-often
http://ceaccp.oxfordjournals.org/content/7/5/171.full

- Reservoir = drug concentration is established, drug moves further into the skin, into the capillaries, and then into the circulation
- There is a time to reach steady state of plasma concentration

Effect of drug characteristics

The properties of a drug that enable good penetration through the stratum corneum can be deduced from the equation for steady-state flux.[2] When the cumulative mass of a diffusant, m, passing per unit area through a membrane is plotted, after time t, the graph approaches linearity and the slope yields the steady flux dm/dt,

$$\frac{d_m}{d_t} = \frac{DC_oK}{h}$$

where D is the diffusion coefficient, C_o the constant concentration of drug in donor solution, K the partition coefficient of solute between membrane and bathing solution, and h the thickness of the membrane.

Therefore, for a drug to penetrate well, it should have low molecular mass (high D), adequate solubility in oil (high C_o), and a moderately high partition coefficient. All of the drugs currently available in patch formulation share three features that enable administration through a convenient area of skin: molecular mass <500 Da; high lipophilicity; and low required daily dose (<2 mg). The comparison of the physicochemical properties of fentanyl, buprenorphine, and morphine (Table 2) demonstrates why fentanyl and buprenorphine are suitable for transdermal delivery.[3]

Parameters that we have to consider:

- Diffusing peptide must not affect the adhesive and vice versa
- Skin compatibility, chemical compatibility
- Tack, peel adhesion, skin adhesion and cohesive strength
- Hydration of skin
    - Tissue swells when skin is saturated with water and its permeability increases = this would be an important factor to increase penetration
- Temperature
- Diffusion coefficient
    - Diffusion speed of molecules depend on the state of matter in the medium
- Drug concentration
    - Drug permeation usually follows the Fick's law. The flux of solute is proportional to the concentration gradient across the entire barrier phase
- Partition coefficient
    - Important in establishing flux of drug through stratum corneum
- Molecular size

Permeability Coefficient Is the Critical Predictor of Transdermal Delivery

Transport = Flux = (mg/cm^2/sec) = P x A x (C_d – C_r)

Permeability Coefficient = P = $\frac{D \times K}{h}$ (cm/sec)

Where A = Surface area of patch

D = Diffusivity of drug in membrane (skin)

K = Partition coefficient (patch/skin)

C = Concentration in donor or receptor (patch or skin)

h = Thickness of membrane (skin)